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Role of the novel cannabinoid receptor GPR55 in islets of Langerhans

A thesis submitted by
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for michelle

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Doing a PhD seems to be a daunting task for some, but I have enjoyed every day of this long ride. Regardless the up and down I went through, my proudest achievement is to have so many people supporting and encouraging me throughout this journey:

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ABSTRACT

Previous studies in our lab have reported that CB1 and CB2 cannabinoid receptors are expressed by islet β -cells where they regulate insulin secretion. A novel cannabinoid receptor, GPR55, is also activated by cannabinoids, but its physiological role in islets of Langerhans has not been established: this was investigated in this thesis, which described the expression and function of this novel receptor in islets.

Initial studies of this PhD project demonstrated that GPR55 is expressed at both mRNA and protein levels in isolated mouse and human islets. Pharmacological activation of GPR55 using either a pharmacological agonist (O-1602) or the putative endogenous agonist lysophosphatidylinositol (LPI) elevated intracellular Ca^{2+} levels ($[\text{Ca}^{2+}]_i$) in Fura-2-loaded mouse and human islet cells. In addition, both agonists also stimulated insulin secretion from perfused mouse and human islets at a sub-stimulatory glucose concentration (2mM) and potentiated glucose-stimulated insulin secretion. The stimulatory effects of O-1602 were abolished in islets isolated from GPR55 knockout mice, while the LPI-induced effects on $[\text{Ca}^{2+}]_i$ and insulin secretion were maintained following GPR55 deletion.

The second phase of this PhD project demonstrated that cannabidiol (CBD), which competes with agonists at GPR55 receptors and is commonly used as a GPR55 antagonist, unexpectedly increased $[\text{Ca}^{2+}]_i$ in mouse islet cells. Stimulatory effects in response to CBD were also observed in perfusion experiments using isolated mouse and human islets, with observations of both initiation of insulin secretion at 2mM glucose and potentiation of glucose-stimulated insulin secretion. The effects of CBD on insulin secretion were also observed in experiments using islets isolated from GPR55 knockout mice.

The effects of pharmacological regulation of GPR55 on islet apoptosis were also investigated. Exposure of mouse islets to O-1602 and LPI reduced basal apoptosis and these agents also promoted islet survival in the presence of cytokines, and the GPR55 ‘antagonist’ CBD also reduced apoptosis in the absence and presence of cytokines. In addition, islets from GPR55 knockout mice showed elevations in basal and cytokine-induced apoptosis compared to wildtype mouse islets.

In conclusion, the studies carried out during this PhD project indicate that GPR55 is expressed by islets and its activation stimulates increases in intracellular Ca^{2+} and insulin secretion, and promotes islet survival. Studies using islets isolated from GPR55 knockout mice have shown that the effect of O-1602 is dependent on the presence of GPR55, but LPI and CBD exert GPR55-independent effects in islets. These results suggest that GPR55 plays an important role in the regulation of islet physiology, and that it may contribute to the peripheral regulation of energy balance and glucose homeostasis by cannabinoids.

ABBREVIATIONS

<u>Abbreviation</u>	<u>Definition</u>
AA	arachidonic acid
Ab	antibody
AC	adenylate cyclase
Ach	acetylcholine
ADP	adenosine diphosphate
AEA	anandamide
2-AG	2-arachidonoyl glycerol
Ag	antigen
AKAP	A-kinase anchor protein
AM251	N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide
AS160	Akt substrate 160
ATF-2	activating transcription factor 2
ATP	adenosine triphosphate
B ₀	maximum binding
bp	base pair
BSA	bovine serum albumin
°C	degrees centigrade
Ca ²⁺	calcium ion
CaM	Calmodulin
CaMK	Ca ²⁺ /calmodulin-dependent kinase
cAMP	cyclic adenosine 3,5-monophosphate
CB1	cannabinoid receptor 1
CB2	cannabinoid receptor 2
CBD	cannabidiol
cDNA	complementary DNA
CNS	central nervous system
CO ₂	carbon dioxide
cpm	counts per minute
CREB	cAMP responsible element binding protein
DAG	diacylglycerol
DI H ₂ O	deionised water

DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphates
DPP-4	dipeptidyl peptidase-4
dsRNA	double-stranded RNA
DTT	dithiothreitol
ECS	endocannabinoid system
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Epac	cAMP-activated GTP-exchange factor
ER	endoplasmic reticulum
FBS	fetal bovine serum
Fura-2 AM	Fura-2 acetoxymethyl derivative
GDP	guanosine bisphosphate
GLP-1	glucagon-like peptide-1
GLUT-2	glucose transporter-2
GLUT-4	glucose transporter-2
GPCR	G-protein-coupled receptor
GPR55	G-protein receptor 55
GSK	glycogen synthase kinase
GTP	guanosine triphosphate
HCl	hydrochloric acid
HEK293	human embryonic kidney 293 cell
IFN- γ	interferon gamma
IL-1 β	interleukin-1 beta
IP ₃	inositol 1,4,5,-trisphosphate
IP ₃ R	inositol 1,4,5-trisphosphate receptor
IRS	insulin receptor substrate
K ⁺	potassium ion
K _{ATP}	ATP-sensitive potassium channel
KCl	potassium chloride
kDa	kilodalton
Kir6.2	inward rectifier K ⁺ channel
KO	knockout

LADA	latent autoimmune diabetes of adulthood
LPI	L- α -lysophosphatidylinositol
MAP-2	microtubule-associated protein-2
MAPK	mitogen-activated protein kinase
MIN6	mouse insulinoma cell line derived by targeted expression of the SV40
MLCK	myosin light chain kinase
MODY	Maturity-onset diabetes of the young
MOPS	3[N-morpholino]propanesulphonic acid
mRNA	messenger RNA
mw	molecular weight
NFAT	nuclear factor of activated T-cells
NIDDM	non-insulin-dependent diabetes mellitus
NPY	neuropeptide Y
OEA	oleoylethanolamine
p	probability
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDE	cyclic nucleotide phosphodiesterase
PDK	phosphoinositide-dependent kinase
PEA	phenethylamine
PEG	polyethylene glycol
PH	pleckstrin homology
PI	phosphatidylinositol
PI3K	phosphatidylinositol-3-kinase
PIP ₂	phosphatidylinositol 4,5-bisphosphate
PIP ₃	phosphatidylinositol-3,4,5-trisphosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PLA ₂	phospholipase A ₂
PLC	phospholipase C
PMSF	phenylmethylsulphonyl fluoride
PP	pancreatic polypeptide
PPAR γ	peroxisome proliferator-activated receptor- γ
PVDF	polyvinylidene fluoride

RBD	Rho binding domain
RIA	radioimmunoassay
Rim	Rab-interacting molecule
RNA	ribonucleic acid
ROCK	Rho-associated kinase
RRP	readily releasable pool
RT	reverse transcription
RyR	ryanodine receptor
SDS	sodium dodecylsulphate
SEM	standard error of the mean
SGLT-2	sodium-glucose transport protein 2
SNAP-25	synaptosomal-associated protein of 25kDa
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SUR1	ATP-sensitive sulphonylurea receptor
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TCA	tricarboxylic acid
TNF- α	tumour necrosis factor alpha
TORC2	transducer of regulated CREB activity 2
tRNA	transfer RNA
TRPV	transient receptor potential vanilloid receptor
Tris	tris(hydroxymethyl)aminomethane
TZD	thiazolidinedione
VAMP-2	vesicle-associated membrane protein 2
VGCC	voltage-gated Ca ²⁺ channel
v/v	volume for volume
WHO	World Health Organization
w/v	weight for volume

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Chapter 1 Introduction

CHAPTER 1 INTRODUCTION

1.1 Islets of Langerhans

The pancreas is an important mammalian organ located in the posterior abdominal wall. It is structurally and functionally divided into two parts, the exocrine and the endocrine pancreas. The exocrine pancreas forms the main part of the pancreas and consists of clusters of cells known as acini, which are responsible for secreting digestive enzymes such as amylase, trypsin, and chymotrypsin into the gut. The endocrine pancreas is scattered throughout the exocrine tissues in the form of aggregates of endocrine cells known as islets of Langerhans, named after the German anatomist Paul Langerhans who discovered them. Islets make up 1-2% of the pancreatic mass and are responsible for regulating plasma glucose concentrations.

Each islet contains four main types of cells and they are classified according to their function: the α -cells secrete glucagon, a hormone involved in carbohydrate metabolism; the β -cells secrete insulin that plays a vital role in regulating glucose homeostasis by decreasing plasma glucose levels; the δ -cells secrete somatostatin to regulate α - and β -cells; and the γ -cells produce pancreatic polypeptide that is suggested to regulate food intake and gastric emptying (Katsuura et al., 2002). Another cell type, the ϵ -cell, has also been identified in islets and is found to secrete ghrelin (Wierup et al., 2002) and amylin (Kruger et al., 2007). However, the exact functions of these islet-derived hormones are still uncertain. Islets are highly vascularised and receive 10 times the amount of blood than cells in the exocrine tissues in order to allow for greater nutrient and hormone exchange (Jansson and Hellerstrom, 1983). In addition, it is reported that there is interspecies variability in cell composition and islet structure (Wieczorek et al., 1998). Thus, it has been shown that rodent islets are comprised of approximately 77% β -cells and 18% α -cells (Orci and Unger, 1975), while human islets contain fewer β -cells (55%) and more α -cells (38%) (Cabrera et al., 2006). δ -cells and γ -cells constitute minority cell types in islets from all species, making up 8-10% and 3-5% of the islet mass, respectively, in rodents and human (Cabrera et al., 2006). Furthermore, cells in mouse

islets are clearly segregated as β -cells clustered at the core surrounded by a ring of α - and δ -cells. In contrast, different cell types are found integrated together in human and monkey islets (Figure 1.1.1) (Cabrera et al., 2006). The variability in islet topography allows unique cell-to-cell interactions, for example via gap junctions or paracrine signalling, resulting in different islet functions. For example, oscillations in membrane potential are coordinated in rodent β -cells because they are clustered, while they are not synchronised in human β -cells since they are segregated. This may explain some of the differences in changes in intracellular calcium levels and insulin secretory profiles between rodent and human islets (Cabrera et al., 2006). These species differences in islet cell composition and topography may reflect evolutionary adaptation to different dietary habits or other environmental factors.

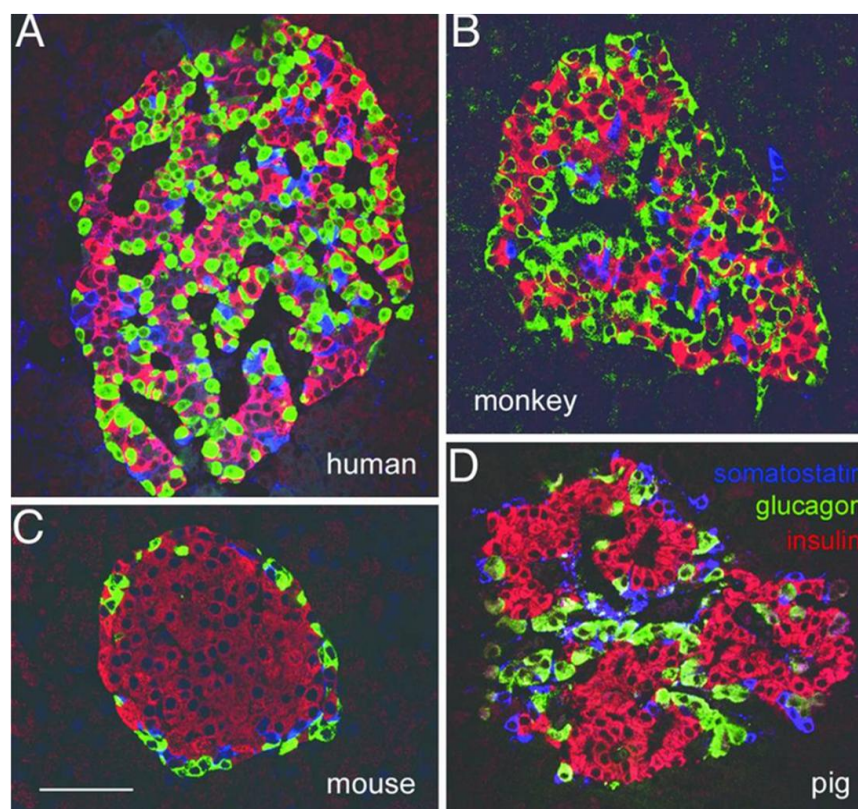


Figure 1.1.1 Interspecies variability in cell composition and islet structure.

Islets from four sources (human, **A**; monkey, **B**; mouse, **C**; pig, **D**) were analysed using confocal microscopy and multiple immunofluorescent labelling. Insulin- (red), glucagon- (green) and somatostatin-expressing cells (blue) are seen in all islets, with insulin-containing cells being the most abundant. In mouse islets, insulin-containing cells are seen clustered at the core, with few glucagon- and somatostatin-expressing cells surrounding them. In contrast, insulin-, glucagon- and somatostatin-expressing cells distribute throughout the human and monkey islets. Pig islets are made up of several smaller subunits that have similar architecture to mouse islets. Scale bar, 50 μ m, figure taken from (Cabrera et al., 2006).

1.1.1 Regulation of glucose storage by insulin

Plasma glucose levels are closely regulated and maintained within a range of 3 to 5 mM in healthy individuals despite their ever-changing feeding and fasting patterns. This tight control is managed by the central nervous system (CNS) and peripheral tissues including liver, skeletal muscle, gastrointestinal tract, adipose tissue and endocrine pancreas. Elevated plasma glucose levels, neuronal signals from the parasympathetic nervous system, and non-nutrient secretagogues stimulate insulin release from islet β -cells. Insulin serves as the primary regulator of blood glucose in the body by increasing glucose uptake into muscle and adipose tissue, promoting glucose storage in muscle, liver and adipose tissues, and by inhibiting glucose production in the liver.

Insulin exerts its action via heterotetrameric insulin receptors of the receptor tyrosine kinase family on insulin-sensitive tissues. Binding of insulin induces a conformational change in the extracellular α subunits of the insulin receptors leading to the activation of the intracellular β -subunits by phosphorylation on multiple tyrosine residues. This allows recruitment and tyrosine phosphorylation of the insulin receptor substrates (IRS) (Schlessinger, 2000). There are four main members of the IRS protein family (Burks and White, 2001): IRS-1 and -2 are ubiquitously expressed (Sun et al., 1991; Sun et al., 1995), while IRS-3 is believed to be preferentially expressed in adipocytes (Lavan et al., 1997) and IRS-4 is primarily found in the CNS (Fantin et al., 1999). Tyrosine phosphorylated IRS proteins recruit downstream effectors, for example, IRS-1 and IRS-2 becomes a docking site for the regulatory subunit p85 of class IA phosphatidylinositol 3-kinase (PI3K). The IRS-p85 complex then recruits and activates the catalytic subunit p110 α of PI3K, which catalyses the conversion of phosphatidylinositol-4,5-bisphosphate (PIP₂) to phosphatidylinositol-3,4,5-trisphosphate (PIP₃) in the plasma membrane. Increased PIP₃ levels leads to the accumulation of signalling proteins containing pleckstrin homology (PH) domain at the cell surface (Cantley, 2002). Association with PIP₃ activates phosphoinositide-dependent kinase 1 (PDK1) to phosphorylate the serine-threonine kinase

Akt/PKB, another signalling protein recruited via its PH domain. Together with phosphorylation by transducer of regulated CREB activity 2 (TORC2), PDK1 stimulates the catalytic activity of Akt/PKB, which phosphorylates a wide range of downstream proteins, such as Akt substrate 160 (ASK160), glycogen synthase kinase 3 (GSK3) and mammalian target of rapamycin complex 1 (mTORC1), leading to the cellular responses to insulin (Manning and Cantley, 2007).

Glucose uptake

One of the main insulin-mediated cellular responses is the stimulation of GLUT4 translocation to the cell surface of adipose and muscle cells for glucose uptake. Adipose and muscle cell-specific glucose transporter GLUT4 is one of the 13 glucose transporter proteins encoded in the human genome. It catalyses transport of hexoses across the cell membrane in an energy-independent manner (Huang and Czech, 2007). Akt/PKB-induced AS160 phosphorylation directly regulates the translocation of GLUT4 from the cytoplasm to the plasma membrane where it facilitates the entry of extracellular glucose to reduce plasma glucose concentrations (Leney and Tavaré, 2009).

Glucose storage

In addition to promoting glucose uptake, insulin stimulates glycogen accumulation via Akt/PKB-induced GSK3 inhibition and consequent glycogen synthase activation in muscle and liver (Cohen and Frame, 2001). Insulin also profoundly inhibits the production and release of glucose in the liver by blocking gluconeogenesis and glycogenolysis via regulating the activities of a set of metabolic enzymes, such as phosphoenolpyruvate carboxykinase, glucose-6-phosphatase and glycogen phosphorylase, by transcriptional and nontranscriptional mechanisms (Barthel and Schmoll, 2003; Hartmann et al., 1987). In adipocytes, insulin promotes glucose storage in the form of triacylglycerol by increasing activation of lipid

synthetic enzymes. Furthermore, insulin inhibits lipolysis, primarily through inactivation of the insulin-sensitive lipases (Saltiel and Kahn, 2001).

1.1.2 Regulation of insulin secretion

In order to maintain constant blood glucose levels, islet β -cells need to secrete the appropriate amount of insulin in response to nutrient and non-nutrient stimulations. Indeed, β -cells are well-equipped with different tools to sense the external factors and to tune their insulin secretion levels. Healthy and functional β -cells are essential for the secretion of this hormone to ensure the precise regulation of glucose homeostasis.

This insulin gene in the β -cells encodes for a 110-amino acid precursor molecule known as preproinsulin. This polypeptide is translocated across the rough endoplasmic reticulum (ER) membrane into the lumen, where it is post-translationally modified to yield proinsulin. Proinsulin consists of the A and B chains of insulin (21 and 30 amino acid residues, respectively) joined by a 31-amino acid C-peptide, which blocks the active site of proinsulin whose biological activity is only about 5% of insulin. Aided by ER chaperone proteins, proinsulin undergoes folding and formation of two disulphide bonds between A and B chains of insulin and one intra-chain bond of A chain for a matured three dimensional conformation. The folded proinsulin is transported from the ER to the Golgi apparatus, where proinsulin enters immature secretory vesicles. The conversion from proinsulin to insulin by proteolytic cleavage of C-peptide is initiated by a decrease in pH from the trans-Golgi network to the secretory vesicles (Davidson et al., 1988). C-peptide is removed via the Ca^{2+} -dependent actions of endopeptidases, prohormone convertases 2 and 3, and carboxypeptidase H (Hutton, 1994). Insulin and C-peptide are then stored in these vesicles, where insulin is bound to Zn^{2+} to form stable crystalline insulin hexamers (Howell et al., 1969). Other β -cell secretory products, such as islet amyloid polypeptide (Nishi et al., 1990), are stored together and released simultaneously with insulin during exocytosis in response to stimulation.

1.1.2.1 Glucose-induced insulin secretion

β -cells respond to many nutrients, but they primarily respond to glucose in the blood due to their highly responsive coupling mechanisms. Oral intake of 75g of glucose by human subjects initiates an increase in plasma insulin concentration from 20-30 pM to 250-300 pM in 30 minutes (Chang and Goldberg, 1978). Islet β -cells do not have membrane-bound glucose receptors, but are equipped with insulin-independent glucose transporters to take up glucose (GLUT-2 in rodents; GLUT-1, 2 and 3 in humans) (De Vos et al., 1995; Richardson et al., 2007). Upon entering β -cells, glucose triggers a series of metabolic events to induce insulin secretion. In brief, glucose is phosphorylated by the rate-limiting enzyme glucokinase and phosphorylated glucose undergoes glycolysis to produce the end product pyruvate, which is fed into the tricarboxylic acid (TCA) cycle in mitochondria. The resulting increase in the cytosolic ATP/ADP ratio leads to the closure of ATP-sensitive K^+ channels (K_{ATP} -channels) by binding to the Kir6.2 subunits. This results in decreased K^+ efflux and plasma membrane depolarisation, followed by the opening of voltage-gated Ca^{2+} channels (VGCCs). An influx of Ca^{2+} activates exocytosis of secretion-ready insulin-containing vesicles (readily releasable pool, RRP) docked in close proximity to the VGCCs. Exocytosis in β -cells takes place in a similar mechanism to the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-regulated synaptic vesicle release. The fusion process between the insulin vesicles and the plasma membrane requires the assembly of SNARE protein complex including the plasma membrane proteins syntaxin and synaptosomal-associated protein of 25kDa (SNAP-25), and vesicle-associated membrane protein 2 (VAMP-2) on the vesicle membrane (Rorsman and Renstrom, 2003). This allows the insulin vesicles to dock to the Ca^{2+} -channels and fuse membranes after localised elevation in $[Ca^{2+}]_i$. Synaptotagmin proteins V or VII of the SNARE complex are responsible for sensing Ca^{2+} (Gut et al., 2001). A small GTP-binding protein Rab3A and its interaction partner Rab3-interacting molecule (Rim) on the insulin vesicles helps the SNARE complex to associate with synaptotagmin, as well as the Ca^{2+} channels (Rorsman and Renstrom, 2003). In addition, a study has shown that calpain-10, a Ca^{2+} -

dependent protease, binds to the SNARE complex and triggers SNAP-25 proteolysis, a process which leads to exocytosis in β -cells (Marshall et al., 2005). The cellular signalling pathway for insulin secretion summarised above is conventionally known as the K_{ATP} channel-dependent pathway (Figure 1.1.2). It has also been reported that a subset of hypothalamic neurons use the same mechanisms as β -cells to sense and respond to change in extracellular glucose concentrations (Squires et al., 2005).

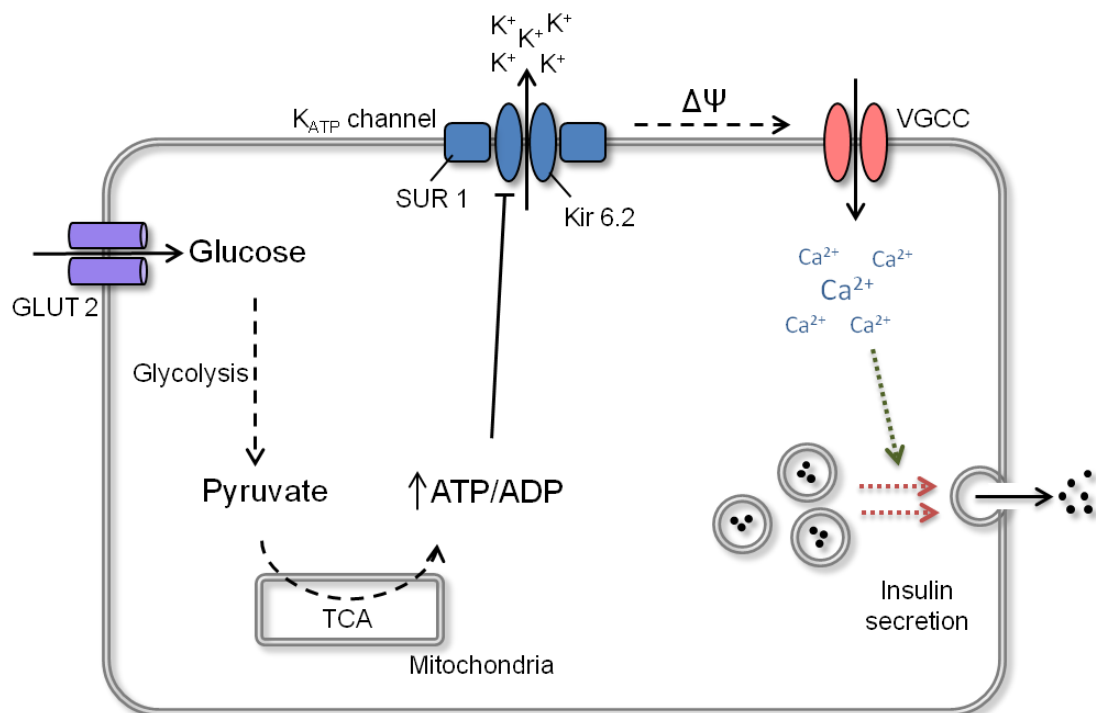


Figure 1.1.2 Schematic representation of the K_{ATP} channel-dependent pathway of glucose-induced insulin secretion.

Glucose is taken up by β -cells via GLUT2 transporters and phosphorylated by glucokinase after entering the cell. Phosphorylated glucose is then processed through glycolysis to pyruvate, which is converted to ATP via the tricarboxylic acid (TCA) cycle in mitochondria. The elevated ATP/ADP ratio results in the closing of K_{ATP} channels via binding to their Kir6.2 subunits. This leads to decreased K^+ efflux, membrane depolarisation (Ψ) and the opening of voltage-gated Ca^{2+} channels (VGCCs). Influx of Ca^{2+} through VGCCs initiates a rapid increase of $[Ca^{2+}]_i$. Ca^{2+} molecules interact with Ca^{2+} sensing proteins within SNARE complexes (synaptotagmins and calpain-10, not shown), which facilitates the fusion process between the plasma membrane and insulin containing vesicles.

Insulin secretion in response to elevated glucose exhibits a characteristic biphasic pattern that consists of a rapid but transient insulin release (first phase), that is followed by a sustained output (second phase). Experiments to date suggest that insulin vesicles exist in distinct functional pools and orderly release of these pools results in the observed biphasic insulin secretory profile. It is believed that the first phase involves rapid release of a small number of docked vesicles in the RRP (1-5% of all vesicles) straight after stimulation. When the RRP is depleted, vesicles from the reserve pool (95-99%) mobilise to the RRP, but they have to undergo a series of ATP-, Ca^{2+} , time- and temperature-dependent reactions known as priming (Rorsman and Renstrom, 2003). This subsequent supply of new vesicles for release is responsible for the sustained second phase of insulin secretion. In addition to the K_{ATP} -dependent pathway, various cellular signalling pathways involving different second messengers and their coupling enzymes have also been implicated in the biphasic nature of insulin secretion.

In addition to nutrients, islet β -cells are also subject to regulation by hormones, which are delivered to the cells via the circulation or released by adjacent cells within the islets, resulting in a paracrine modulation of insulin secretion. Thus, islet hormones such as glucagon and somatostatin can regulate insulin output from β -cells, with stimulatory and inhibitory effects, respectively, and insulin secretion may also be modified by neurotransmitters and gastrointestinal incretins (Jones and Persaud, 2010). Many of these agents regulate insulin secretion through the signalling cascades described below following interaction with their target receptors on β -cells.

1.1.2.1.1 Role of CaMKs in the regulation of insulin secretion

In addition to the SNARE complex, β -cells express a variety of Ca^{2+} -sensitive enzymes that may be involved in sensing and responding to changes in $[\text{Ca}^{2+}]_i$. It is well-established that Ca^{2+} /calmodulin-dependent protein kinases (CaMKs) are activated in the presence of Ca^{2+} and

the Ca^{2+} -binding protein calmodulin (CaM). CaM detects increases in $[\text{Ca}^{2+}]_i$ and binds to free Ca^{2+} to form Ca^{2+} /CaM complexes, which mediate downstream Ca^{2+} -initiated responses by regulating the functions of CaMKs. CaMKs modulate their downstream effects by phosphorylating endogenous substrates on serine/threonine residues. A number of subtypes of CaMKs are expressed by β -cells: myosin light chain kinase (MLCK) and multifunctional CaMK II, III and IV (Ban et al., 2000; Jones and Persaud, 1998). Most investigations of CaMKs in β -cells have focused on CaMK II and data from a number of groups suggests that CaMK II is involved in glucose-induced insulin secretion (Figure 1.1.3) (Jones and Persaud, 1998). Identified substrates of CaMK II in β -cells include synapsin-1 and microtubule-associated protein-2 (MAP-2) (Easom, 1999), which are involved in vesicle trafficking and docking, and ryanodine receptor 2 (RyR_2) (Okamoto and Takasawa, 2002), which is important to maintain sustained Ca^{2+} oscillations via Ca^{2+} release from the ER.

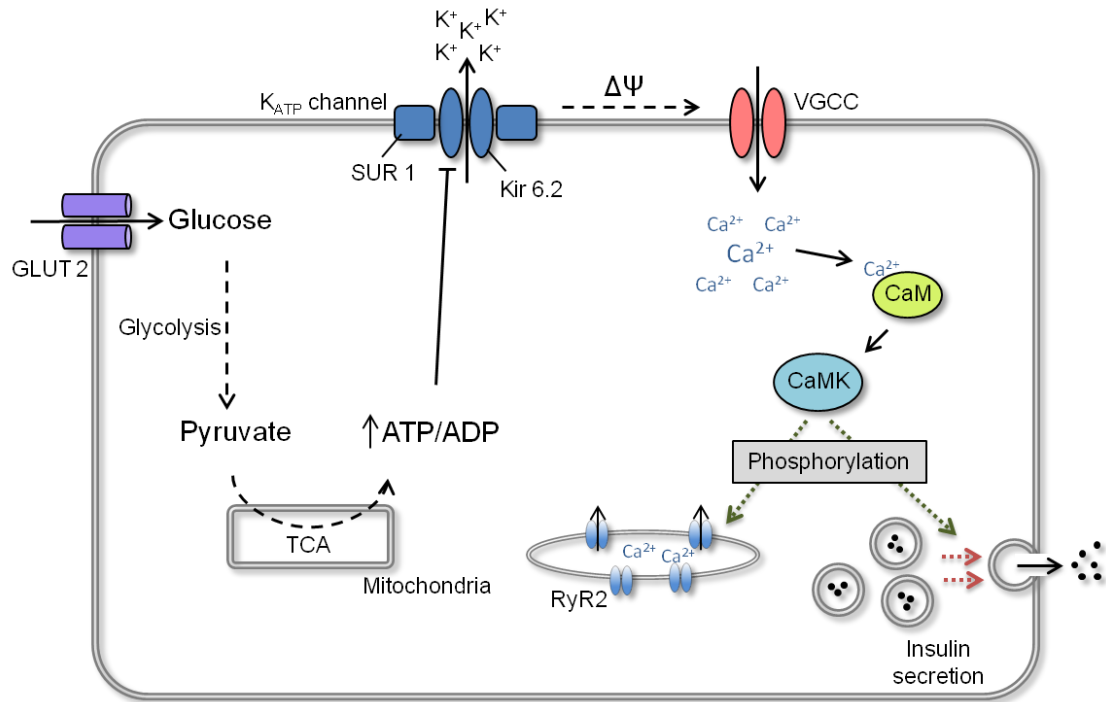


Figure 1.1.3 Schematic representation of CaMK signalling in insulin secretion.

The K_{ATP} channel-dependent pathway in β -cells leads to elevations in $[Ca^{2+}]_i$ which activates Ca^{2+} /calmodulin-dependent protein kinases (CaMKs) via binding to calmodulin (CaM). CaMKs, such as CaMK II, phosphorylate ryanodine receptor 2 (RyR₂) resulting in sustained Ca^{2+} release from the ER. CaMK II further phosphorylates synapsin-1 and microtubule-associated protein-2 (not shown) to promote insulin exocytosis.

1.1.2.1.2 Role of the PLC/PKC pathways in the regulation of insulin secretion

Glucose also activates Ca^{2+} -dependent phospholipase C (PLC) in β -cells (Biden et al., 1987). PLC breaks down membrane-bound phosphatidylinositol-4,5-bisphosphate (PIP₂) into cytosolic inositol 1,4,5-trisphosphate (IP₃) and membrane-bound diacylglycerol (DAG). Increased intracellular IP₃ activates IP₃ receptors located on the ER to allow Ca^{2+} release which has been implicated in contributing to glucose-induced $[Ca^{2+}]_i$ elevation and both phases of insulin release (Dufer et al., 2007; Wiederkehr and Wollheim, 2008). On the other hand, DAG recruits and activates of protein kinase C (PKC), which is a serine-threonine family of enzymes that has three main classes: the conventional PKCs, which include isoforms α , β and

γ , are activated by Ca^{2+} and DAG; the novel PKCs, consisting of isoforms δ , ϵ , η and θ , are regulated by DAG but not Ca^{2+} ; and the atypical PKCs, isoforms ζ , ι and μ , are considered to be Ca^{2+} - and DAG-independent (Idris et al., 2001). It has been shown that glucose is able to induce PKC translocation to the plasma membrane of β -cells (Persaud et al., 1989). The secretory response to glucose can also be enhanced by pharmacologically suppressing DAG breakdown (Zawalich et al., 1989). Moreover, exposure of islets to glucose results in the activation of an established downstream substrate of PKC, the myristoylated alanine-rich C kinase substrate (MARCKS) (Calle et al., 1992). Various proteins important to the secretory response in β -cells have also been identified as substrates of activated PKC, including the sulphonylurea receptor-1 (SUR1) subunit of the K_{ATP} channel (Inagaki et al., 1995), VGCCs (Seino, 1995), cytosolic phospholipase A_2 (Dunlop and Clark, 1995) and other G protein-dependent signalling cascades (Persaud et al., 1993). On the other hand, it was found that PKC depletion from β -cells did not affect glucose-induced insulin secretion, suggesting that activated PKCs are not necessary for nutrient-induced insulin secretion. Importantly, the Ca^{2+} - and DAG-dependent PKCs are essential mediators in ligand-induced insulin secretion through the activation of G protein-coupled receptors (GPCRs) and the Gq/PLC signalling pathway, such as the response to the cholinergic neurotransmitter acetylcholine (Ach) (Persaud et al., 1993). Furthermore, in addition to its role of activating PKC, DAG is also a precursor of other intracellular messengers including arachidonic acid (AA) and the endocannabinoid 2-arachidonoyl glycerol (2-AG).

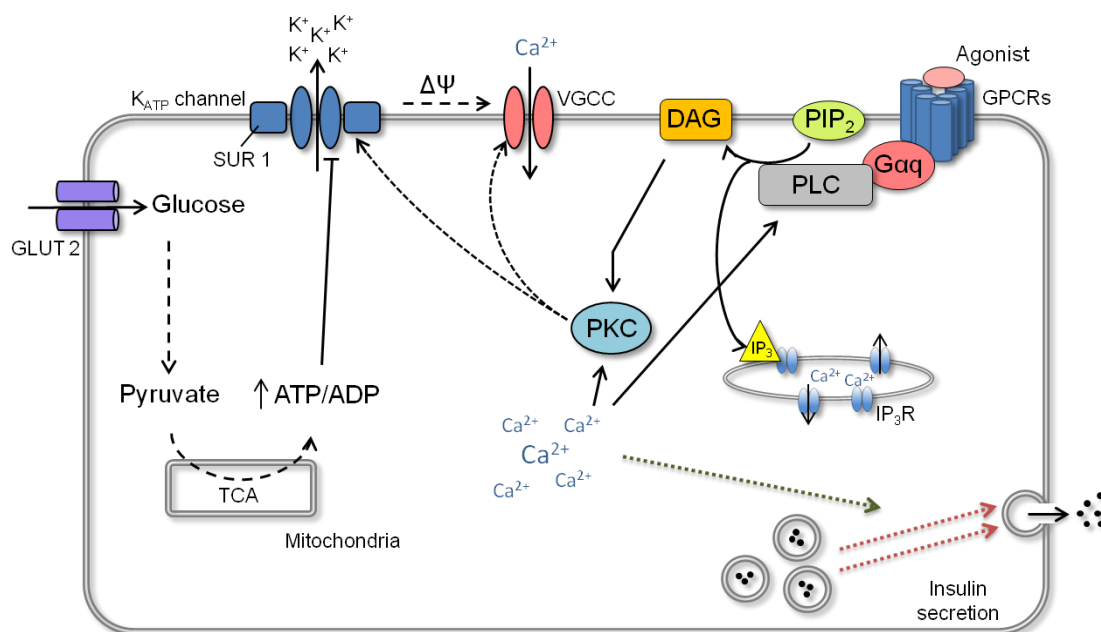


Figure 1.1.4 Schematic representation of PLC/PKC signalling in insulin secretion.

Glucose-induced elevation in $[Ca^{2+}]_i$ activates Ca^{2+} -dependent phospholipase C (PLC), which breaks down phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 diffuses through the cytoplasm and activates IP_3 receptors (IP_3R) on the ER to increase $[Ca^{2+}]_i$ and insulin secretion. DAG activates Ca^{2+} -dependent protein kinase C (PKC) isoforms, which phosphorylate and activate a set of substrates, such as the SUR1 subunit of K_{ATP} channel and VGCCs, to potentiate insulin secretion. A range of secretagogues such as acetylcholine potentiate glucose-induced insulin secretion via G protein-coupled receptors (GPCRs) and the Gq-dependent PLC and PKC signalling pathway.

1.1.2.1.3 Role of cAMP/PKA/Epacs pathways in the regulation of insulin secretion

Cyclic AMP (cAMP) is a universal second messenger regulating a range of cellular responses including insulin vesicle exocytosis in β -cells. Adenylate cyclases (ACs) hydrolyse ATP into cAMP, which is degraded by cyclic nucleotide phosphodiesterases (PDEs). Eight of the nine known membrane-bound AC isoforms are expressed in islets and β -cell lines (Delmeire et al., 2003; Landa et al., 2005; Leech et al., 1999). For cAMP degradation, 25 PDE genes have been identified that encode a superfamily of more than 50 different PDE proteins, with PDE1C, PDE3B, PDE4A, PDE4D, PDE8A and PDE10A being expressed by islets (Szaszak et al., 2008). The activities of ACs and PDEs are regulated by numerous signalling pathways to

govern intracellular cAMP levels. It is well established that cAMP is not considered as an essential messenger mediating the insulinotropic effect of glucose, but it is believed that cAMP can modulate the action of glucose in β -cells (Malaisse and Malaisse-Lagae, 1984; Prentki and Matschinsky, 1987a). Thus, cAMP alone does not stimulate insulin secretion at low glucose (1-2.8mM) (Charles et al., 1973; Charles et al., 1975), although it does enhance both phases of glucose-induced insulin secretion (Takahashi et al., 2002; Wiedenkiller and Sharp, 1983). Studies using PDE inhibitors and manipulation of the PDE genes demonstrated that PDE plays a negative role in both phases of glucose-induced insulin secretion, confirming the importance of cAMP in β -cell functions (Han et al., 1999; Walz et al., 2007). It was also shown that cAMP increases the size of the RRP and promotes insulin vesicle mobilisation (Hisatomi et al., 1996; Renstrom et al., 1997).

cAMP exerts its physiological effects via both protein kinase A (PKA)-dependent and PKA-independent pathways. The PKA family, consisting of type I and II isoforms, are downstream effectors activated by cAMP. Once activated, PKA phosphorylates its substrates on serine/threonine residues to trigger cellular effects with the help from a scaffolding protein, A-kinase anchor protein (AKAP). cAMP-dependent PKA activation has been shown to trigger an increase in the activities of L-type VGCCs by phosphorylating the ion channels (Ammala et al., 1993; Yada et al., 1993) and PKA phosphorylation primes the ryanodine receptor Ca^{2+} channels (RyR) on the ER membrane, so the channels are ready to be activated by Ca^{2+} influx (Islam, 2002). PKA therefore plays an important role in regulating and amplifying Ca^{2+} signals during glucose-induced insulin secretion.

In addition to PKA, cAMP can mediate its physiological effects through several other effector proteins, including cAMP receptor protein (CRP), cyclic nucleotide-gated (CNG) channels and cAMP-activated GTP-exchange factor (Epac) (Seino and Shibasaki, 2005). In β -cells, it was found that cAMP can stimulate exocytosis of insulin vesicles in the presence of PKA

inhibition, suggesting that cAMP regulates insulin secretion in a PKA-independent fashion (Renstrom et al., 1997). Subsequent experiments identified Epac1 and Epac2 expression in β -cells and it was demonstrated that Epac1/2 interacts with the SUR1 subunit of K_{ATP} channels (Ozaki et al., 2000; Shibasaki et al., 2004). It was also shown that Epac2 mediates Ca^{2+} mobilisation via activating RyRs independent of PKA (Kang et al., 2001; Kang et al., 2003). In addition, Epac2 can also regulate insulin vesicle exocytosis through interaction with two other proteins, Rim2 and Piccolo, and their absence results in impaired docking and fusion steps of insulin secretion. Rim2 is known to interact with Rab3, which is involved in the exocytosis process of RRP in β -cells, while Piccolo interacts with Rim2 in a Ca^{2+} -dependent manner, acting as a Ca^{2+} sensor (Fujimoto et al., 2002; Kashima et al., 2001; Ozaki et al., 2000). Epac2, together with Rim2, Piccolo and Rab3, may form a complex that senses intracellular cAMP and Ca^{2+} levels. Although the exact role of cAMP and the Epac/Rim2/Piccolo complex in insulin vesicle exocytosis remains unclear, results published so far indicate that they play a significant role in regulating insulin secretory response to glucose.

It has been suggested that the cAMP signalling system works together with the Ca^{2+} signalling cascade to regulate β -cell function. This was first proposed when Ca^{2+} influx after β -cell membrane depolarisation was reported to induce elevations in intracellular cAMP levels via Ca^{2+} -regulated ACs (Charles et al., 1975). Several isoforms of ACs (type I, III and VIII) and PDEs (PDE1s) contain CaM-binding sites (Taussig et al., 1994; Yan et al., 1995), indicating that cAMP levels can be regulated in a Ca^{2+} /CaM-dependent manner. This is further supported by studies in β -cell lines demonstrating that Ca^{2+} regulates cAMP production (Delmeire et al., 2003; Landa et al., 2005; Leech et al., 1999; Walz et al., 2007). On the other hand, it was described previously that cAMP modulates VGCC activities and Ca^{2+} influx via PKA (Ammala et al., 1993; Yada et al., 1993). cAMP can further regulate $[Ca^{2+}]_i$ in β -cells via RyR-mediated Ca^{2+} -induced Ca^{2+} release via both PKA-dependent and PKA-independent mechanisms (Islam, 2002; Kang et al., 2001; Kang et al., 2003). Hence, it is likely that there is

a dual cAMP and Ca^{2+} signalling pathway playing a significant role in regulating insulin secretion and other physiological functions in β -cells.

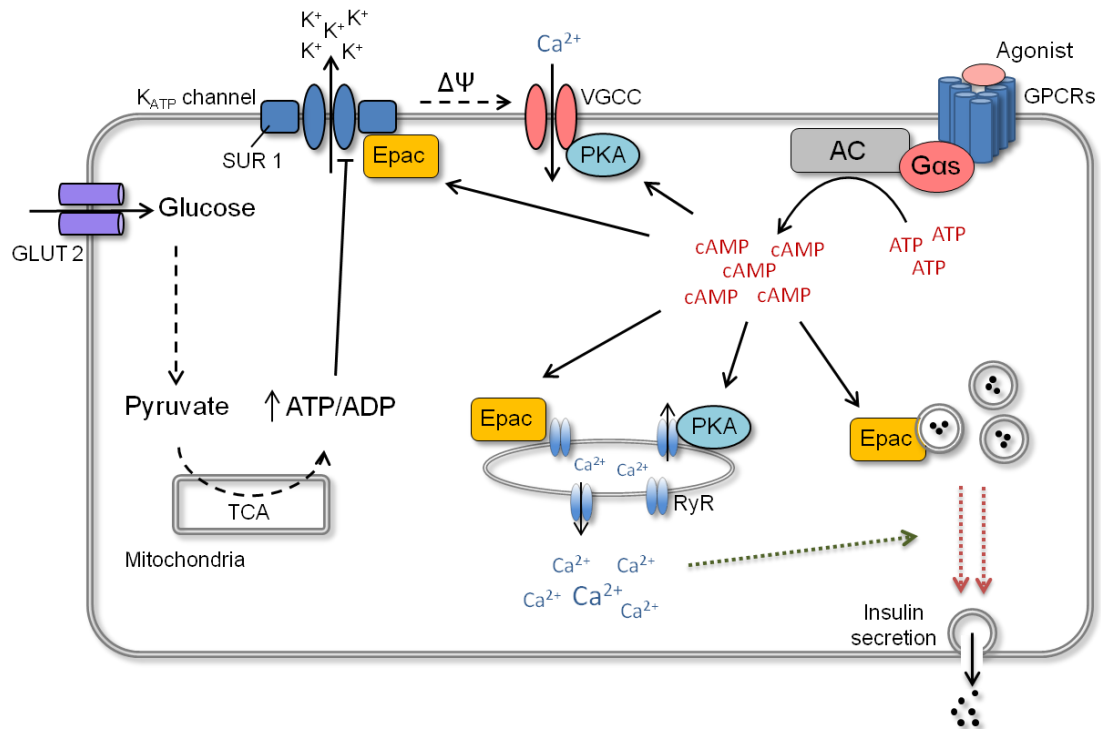


Figure 1.1.5 Schematic representation of cAMP signalling in insulin secretion.

Glucose uptake and metabolism lead to an increase in ATP, membrane depolarisation and opening of voltage-gated calcium channels (VGCCs) in β -cells. Adenylate cyclase (AC), which can be activated by G protein-coupled receptors (GPCRs) via Gs proteins, converts ATP into the second messenger cAMP in a Ca^{2+} -dependent manner. Increases in cytosolic cAMP lead to activations of its downstream effectors, such as protein kinase A (PKA) and cAMP-activated GTP-exchange factor (Epac). PKA, anchored via A-kinase anchor protein (AKAP, not shown) to its substrates, phosphorylates VGCCs and ryanodine receptor (RyR) on the ER membrane to increase $[\text{Ca}^{2+}]_{\text{i}}$. Epac (1 & 2) also interacts with RyR and the SUR1 subunit of K_{ATP} channels to modulate $[\text{Ca}^{2+}]_{\text{i}}$. In addition, Epac2 can facilitate docking and fusion steps of insulin vesicle exocytosis through interaction with two other proteins, Rim2 and Piccolo (not shown). Collectively, cAMP enhances glucose-induced insulin secretion via both PKA-dependent and PKA-independent mechanisms.

1.1.2.2 Regulation of insulin secretion by GPCRs

Glucose-induced insulin secretion from islet β -cells is modulated by other factors to optimise insulin secretion according to the metabolic demand. Many of these factors act through the largest class of cell surface receptors, the G-protein coupled receptor (GPCR) family, and nearly 300 members have been identified in islets (Amisten et al., 2013). Although functions of many of these GPCRs remain unknown in islet biology, the others are known to be regulated by a diverse range of extracellular signals including neurotransmitters, hormones and nutrient factors, such as lipids and peptides. Several of these GPCRs have been examined as potential therapeutic targets to stimulate insulin secretion to treat Type 2 diabetes mellitus, and drugs activating islet glucagon-like peptide-1 (GLP-1) receptor were successfully introduced for clinical use in 2005.

GPCRs are membrane-bound proteins that have seven transmembrane domains connected by various intracellular and extracellular domains. Each GPCR is associated with a membrane-bound heterotrimer protein complex known as GTP-binding proteins (G proteins). The G protein complex consists of α , β and γ subunits. In the inactive form, the receptor-associated α subunit binds to guanosine diphosphate (GDP) and forms a complex with $\beta\gamma$ subunits. Activation of the GPCR results in a conformational change in the α subunit to release GDP in exchange for guanosine triphosphate (GTP). GTP binding leads to the release of the $\beta\gamma$ complex and α and $\beta\gamma$ complexes each regulate a variety of downstream signalling proteins. Each GPCR is associated with an α subunit from four main classes (Gs, Gi, Gq and G12/13) with a total of 20 subtypes. In addition, there have been five β subunits and 13 γ subunits identified to date, allowing various combinations of $\beta\gamma$ complex. Research so far has focused on the regulatory role of α subunits in GPCR-mediated regulation of insulin secretion from β -cells and it remains unclear what role $\beta\gamma$ complexes play in islet biology. The effect of each α subunit subclass on insulin secretion is summarised below and illustrated in Figure 1.1.6.

Gs α subunit

Activation of Gs subunits leads to AC activation and increases in cAMP, thereby potentiating glucose-induced insulin secretion through PKA and Epac (Figure 1.1.5). A wide range of GPCRs signal through the Gs-mediated signalling pathway to stimulate insulin secretion. These include GLP-1 receptor (GLP-1R) (Doyle and Egan, 2007), glucose-dependent insulintropic peptide (GIP) receptor (Flatt, 2008), glucagon receptor (Jiang and Zhang, 2003), β 2-adrenoceptor (McGraw and Liggett, 2005), lipid amide-activated GPR119 (Soga et al., 2005) and many others (Amisten et al., 2013).

Gi α subunit

The Gi subunit acts opposite to the Gs subunit to negatively regulate intracellular cAMP levels via inhibition of ACs. Activation of Gi-coupled GPCRs, therefore, leads to inhibition of insulin secretion. Islet hormone ghrelin secreted by ϵ -cells inhibits insulin secretion in a paracrine manner via activating the Gi-coupled ghrelin receptor (Reimer et al., 2003). Other islet GPCRs associated with Gi subunits include melatonin receptor (Ramracheya et al., 2008) and neurotransmitter receptors, α 2-adrenoceptors (Chan et al., 2001) and neuropeptide Y receptors (Morton and Schwartz, 2001).

Gq α subunit

The Gq subunit stimulates β class of PLC to generate IP₃ and DAG from PIP₂. As previously described in Figure 1.1.4, IP₃ and DAG potentiate insulin secretion via triggering the release of Ca²⁺ from the ER and activating PKC, respectively. Muscarinic acetylcholine receptor subtype 3 (Gautam et al., 2006), cholecystokinin A receptor (Berna et al., 2007), kisspeptin receptor GPR54 (Bowe et al., 2009), GPR40 activated by medium- to long-chain fatty acids (Itoh et al., 2003) are all well-established Gq-coupled GPCRs in islets.

G12/13 α subunit

In contrast to other G subunits, the G12/13 subunit is unusually linked to activation of the Rho small GTPase family, such as Cdc42, Rac and Rho, which are known to play a vital role in actin cytoskeleton dynamics. Cdc42 and Rac both stimulate insulin secretion from β -cells (Kowluru et al., 1997; Li et al., 2004; Nevins and Thurmond, 2005), while Rho and its effector Rho-associated kinase (ROCK) were suggested to inhibit insulin vesicle exocytosis (Hammar et al., 2009). It remains unclear how the G12/13 subunit may regulate insulin secretion and research to date has not identified any GPCRs that are coupled to G12/13 in β -cells. The function of this class of α subunit in islet biology remains to be explored.

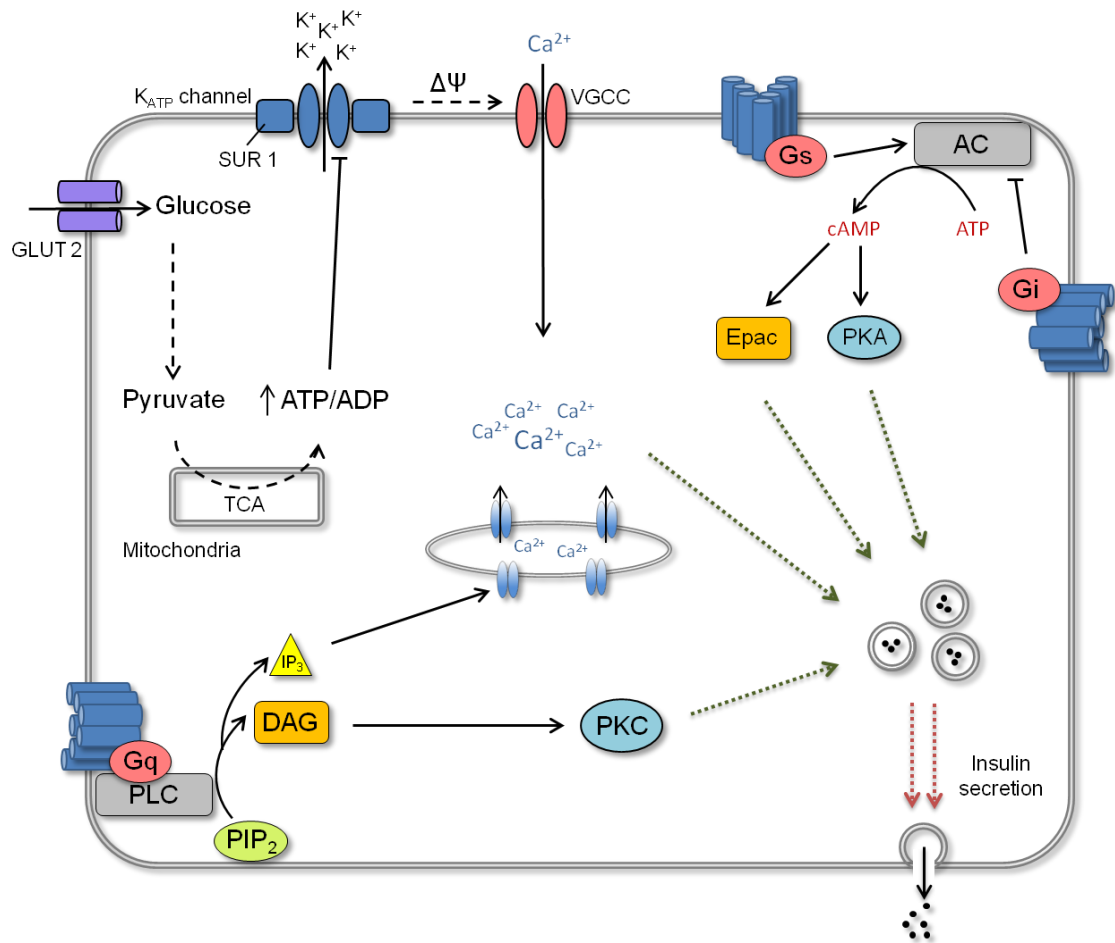


Figure 1.1.6 Schematic representation of GPCR-stimulated pathways in insulin secretion.

Extracellular factors, such as neurotransmitters, hormones and nutrient factors, potentiate or inhibit glucose-induced insulin secretion from β -cells via GPCRs. Each GPCR is specifically coupled to one of the α subunits to induce its downstream effects. Four main types of α subunits (Gs, Gi, Gq and G12/13) exist. The Gs subunit is coupled to activation of ACs and accumulation of cAMP within the cell, which in turn potentiates insulin secretion via PKA and Epac. The binding of agonists to Gi-coupled GPCR inhibits insulin secretion via inhibition of ACs. On the other hand, the Gq subunit is coupled to PLC/PKC pathway to stimulate insulin secretion. In addition, the role of G12/13 subunits in the regulation of insulin secretion remains unknown (not shown).

1.1.3 Regulation of β -cell mass

The amount of insulin that can be secreted by β -cells is not only determined by their secretory capacity, but is also governed by β -cell mass. It is hypothesised that β -cell mass regulation is an important tool to maintain energy homeostasis via changes in insulin supply (Rhodes, 2005). For example, research in rodents has shown that during pregnancy the maternal β -cell population can dramatically increase to meet the extra metabolic demand (Parsons et al., 1992; Sorenson and Brelje, 1997). Although it is not possible to make the same observations in humans, it is thought that up-regulation of β -cell mass also occurs during pregnancy and it is highly adaptive to other changes in metabolic demand, such as obesity and insulin resistance in humans.

There are three independent cellular mechanisms that are regarded to be responsible for maintaining β -cell mass: proliferation (replication of existing β -cells), neogenesis (transformation from precursor cells) and apoptosis (programmed cell death). Replication and neogenesis are mechanisms of β -cell expansion, while apoptosis is a mechanism of controlling β -cell populations through programmed cell death. A mathematical model has been proposed to summarise how these three mechanisms work together to regulate β -cell mass: the sum of the rates of β -cell replication and neogenesis, minus the rate of β -cell apoptosis gives the net rate of change in β -cell mass (Rhodes, 2005).

$\text{Proliferation} + \text{Neogenesis} - \text{Apoptosis} = \text{Rate of change of } \beta\text{-cell mass}$
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The contribution made by different mechanisms may vary in response to the different stages of life or changes in metabolic demand. Studies in rodents have suggested that there is an increase in β -cell mass shortly after birth and during childhood and adolescence. β -cell mass stays relatively constant during adulthood and may decrease later in life (Rhodes, 2005).

Increases in β -cell mass in the early stages of life are results from the neonatal burst of β -cell replication and neogenesis, in combination with a modest amount of β -cell apoptosis. After reaching adulthood, only approximately 0.5% of β -cells undergo apoptosis in parallel to β -cell replication and neogenesis at a similar rate to maintain optimised β -cell mass (Bonner-Weir, 2000). In elderly individuals, overall β -cell mass may decrease since the rate of apoptosis may exceed the rate of replication and neogenesis as a result of ageing. On the other hand, β -cell mass dramatically increases during pregnancy and evidence in rodents suggested that this is a result of an increase in β -cell replication stimulated by the pregnancy hormones prolactin and placental lactogen (Sorenson and Brelje, 1997). β -cell mass returns to normal after gestation via a decreased rate of replication coupled with increased apoptosis.

It is important to note that the model of β -cell mass regulation is primarily obtained from studies in rodents, with only minor observations from human (Rhodes, 2005). There are technical difficulties and ethical reasons hampering detailed study of β -cells in human subjects, although it is believed that a similar model occurs in both rodent and human. Some studies have reported species specificity in terms of the relative contribution of each mechanism. For example, it has been suggested that β -cell replication is primarily responsible for maintaining β -cell mass in adult mice (Dor et al., 2004; Georgia and Bhushan, 2004), while both replication and neogenesis are important in humans (Butler et al., 2003).

1.1.3.1 Regulation of β -cell mass by glucose

Glucose itself has been shown to have an effect on β -cell mass dynamics. Studies in rats have reported that glucose infusion resulted in significant increases in β -cell numbers (Paris et al., 2003; pGarofano et al., 1999), and an *in vitro* study using primary rat β -cells demonstrated that glucose was able to promote cell survival by activating synthesis of anti-apoptotic proteins (Hoorens et al., 1996). Further intracellular signalling studies in β -cell lines have revealed that glucose may act through the Ca^{2+} /CaM-dependent protein kinase 4 (CaMK4)-cAMP

responsive element-binding protein (CREB) pathway to regulate β -cell mass (Hennige et al., 2003; Jhala et al., 2003; Liu et al., 2012). Following GLUT-2 mediated entry into β -cells, glucose is metabolised to generate ATP, which leads to closure of K_{ATP} -channels and Ca^{2+} influx. Ca^{2+} , together with the Ca^{2+} -binding protein CaM, activates Ca^{2+} /CaM-dependent protein kinase kinase (CaMKK), which then phosphorylates CaMK4 (Liu et al., 2012). Activated CaMK4 translocates into the nucleus where it phosphorylates CREB. This phosphorylation is responsible for CREB activation which, in turn, binds to the cAMP response element (CRE) on the IRS-2 promoter to increase IRS-2 gene expression (Persaud et al., 2011). IRS-2 maintains β -cell mass by stimulating proliferation and inhibiting apoptosis through modulating the PI3K/Akt pathway and Ras pathway (Rhodes, 2005).

1.1.3.2 Regulation of β -cell mass by GLP-1

GLP-1 and its analogues improve glycaemic control and enhance β -cell mass (Brubaker and Drucker, 2004). GLP-1 activates the Gs-coupled GLP-1 receptor (GLP-1R) that is linked to cAMP signaling cascade (Brubaker and Drucker, 2002). Current investigations on the mechanism of GLP-1-mediated increase in β -cell mass have indicated three potential pathways: enhancement of proliferation, inhibition of apoptosis and stimulation of neogenesis. Treatment of β -cell lines with GLP-1 increased their rate of proliferation *in vitro* and short- and long-term treatment of rodents with GLP-1R agonists resulted in increased numbers of proliferating β -cells (Brubaker and Drucker, 2004). The proliferating pathway downstream of GLP-1R involves multiple metabolically important proteins, including PI3K, Akt/PKB, MAPK and IRS-2 (Buteau et al., 1999; Trumper et al., 2002). In addition, GLP-1 has also been shown to protect β -cells from undergoing apoptosis both *in vivo* and *in vitro* (Li et al., 2003; Wang and Brubaker, 2002). It also increases the survival rate of β -cell lines in response to the challenges of cytokines, peroxide and fatty acids (Hui et al., 2003; Kwon et al., 2004; Li et al., 2003). More importantly, GLP-1 protects freshly isolated human islets from undergoing apoptosis (Farilla et al., 2003). Biochemistry studies have supported that GLP-1 modulates both

proapoptotic (e.g. caspase 3) and antiapoptotic factors (e.g. Bcl-2, Bcl-xL) via PI3K, Akt/PKB and MAPK (Farilla et al., 2003; Hui et al., 2003; Li et al., 2003; Trumper et al., 2002). Another study suggested that GLP-1 mediates its antiapoptotic effect through CREB-induced up-regulation of IRS-2 gene expression (Jhala et al., 2003). Furthermore, GLP-1 induces the expression of transcription factor pancreas-duodenum homeobox (PDX-1) in ductal precursor cells (Stoffers et al., 2000). The PDX1 gene is important for the embryonic development of endocrine pancreas and GLP-1 may induce PDX1 expression to initiate β -cell neogenesis. It has been shown that GLP-1R agonists were able to induce differentiation of human pancreatic progenitor or duct cells to cells with β -cell-like phenotypes (Abraham et al., 2002; Movassat et al., 2002). Findings so far strongly implicate that GLP-1 regulates multiple signaling pathways that contribute to overall increases in β -cell mass.

1.2 Diabetes mellitus

Diabetes mellitus is a chronic disorder characterised by elevated glucose levels in the blood and urine in affected individuals. In the past few decades, diabetes has grown rapidly into a global epidemic affecting nearly 6% of the world population and causing approximately 5.1 million deaths worldwide per annum. As one of the most serious health issues impinging upon the world community, the disease is costing at least 327 billion pounds a year in health expenditure, 11% of total spending on adults (IDF, 2013). Untreated or poorly managed diabetes results in high risks of developing complications such as cardiovascular disease, neuropathy, retinopathy and nephropathy and it has been estimated that 70-80% of mortality among diabetic patients is due to diabetes-related complications (Meetoo et al., 2007). Diabetes is posing a significant financial and emotional burden to the people of most countries.

Diabetes mellitus is classified into two main types: Type 1 diabetes mellitus (T1DM), formerly known as insulin-dependent diabetes mellitus (IDDM) and Type 2 diabetes mellitus (T2DM), which was previously called non-insulin-dependent diabetes mellitus (NIDDM) (Rhodes, 2005). Both types of diabetes involve the failure of the endocrine pancreas to secrete sufficient amount of insulin to meet the body's metabolic demands. There are also two less common subtypes of diabetes known as maturity-onset diabetes of the young (MODY) and latent autoimmune diabetes of adulthood (LADA). MODY occur when mutations are found in autosomal dominant genes disrupting pancreatic β -cell functions (Stride and Hattersley, 2002), while LADA is caused by autoantibody-induced destruction of β -cells in individuals at the age of 30 years or older (Leslie et al., 2006).

It is estimated that 5-10% of diabetic patients suffer from T1DM, with incidence rates varying greatly between countries: from 0.6 incidences/100,000 per year in China to 57.4 incidences/100,000 per year in Finland (IDF, 2013). The majority of T1DM patients are diagnosed during their teenage years and the disease occurs due to self-destruction of β -cells

and loss of insulin secretion. At the time of clinical diagnosis, approximately 60-80% of β -cells have been destroyed by infiltrating immune cells such as macrophages and CD8 T cells (Notkins and Lernmark, 2001). Previous studies have suggested that T1DM may be inherited and a large number of target genes have since been identified (Anjos and Polychronakos, 2004; Eerligh et al., 2004; Eisenbarth, 2007; Van Belle et al., 2010). A particularly strong correlation was proposed between T1DM and HLA class II immune recognition molecule genes (known as IDDM1, insulin-dependent diabetes mellitus locus 1) found on chromosome 6. In addition, Another high risk locus, IDDM2, was found on chromosome 11 which contains the insulin gene promoter, and it is not surprising that mutations in this locus may also contribute to disease susceptibility (Van Belle et al., 2010). It is estimated that IDDM1 and IDDM2 contribute to approximately 42% and 8%, respectively, of the genetic heritable risks of T1DM (Eerligh et al., 2004). Furthermore, CTLA-4 (cytotoxic T lymphocyte-associated 4) gene, which locates on chromosome 2 and regulates T-cell functions, has also been associated with T1DM, as well as other autoimmune diseases (Anjos and Polychronakos, 2004). Other genes that have been suggested to contribute to the progression of T1DM include PTPN22 (protein tyrosine phosphatase non-receptor type 22) and IL2RA (interleukin-2 receptor- α) (Eisenbarth, 2007). On the other hand, there is evidence, such as studies in monozygotic twins indicated that only 13-33% are pairwise concordant for T1DM, suggest that environmental factors also play a critical role in the pathogenesis of T1DM (Knip et al., 2005). Putative environmental triggers for T1DM include viruses, bacteria, cow's milk, wheat proteins and vitamin D deficiency (Van Belle et al., 2010).

T2DM, which affects 90-95% of diabetic patients, is characterised by chronic hyperglycaemia as a result of insulin resistance of peripheral tissues, combined with insufficient insulin secretion from islet β -cells to compensate for the reduced insulin sensitivity. It is estimated that 532 million people will have T2DM by 2030 and the majority of these patients will be between 40 to 59 years old (IDF, 2013). It is generally accepted that T2DM occurs as a result of both

lifestyle and genetic factors. Lifestyle factors that are important to the development and progression of the disease include physical inactivity, high calorie diet, smoking and drinking (Hu et al., 2001). In addition, obesity has been found to be associated with approximately 55% of T2DM incidences (CDC-US, 2004). On the other hand, studies have identified a spectrum of genes that are linked to T2DM. For example, TCF7L2 (transcription factor 7-like 2), which is responsible for regulating proglucagon gene expression, has been suggested to have the strongest link with the disease (Ridderstrale and Groop, 2009). Other candidate genes include KCNJ11 encoding for the Kir6.2 subunit of the K_{ATP} -channel (Gloyn et al., 2003), peroxisome proliferator-activated receptor gamma (PPAR γ) (Altshuler et al., 2000), CAPN10 which encodes a Ca^{2+} -sensitive cysteine protease (Horikawa et al., 2000), and SLC30A8, coding for the zinc transporter ZnT8 (Sladek et al., 2007).

1.2.1 Therapies for diabetes

The more common treatment prescribed to T1DM patients is insulin replacement therapy, which delivers exogenous insulin to compensate for the lack of insulin secretion by β -cells. Exogenously administered insulin regulates glucose production, utilisation and storage thus maintaining normal glycaemia. In addition, islet transplantation therapy may be an option for patients who have severe hypoglycaemia unawareness or who have had a kidney transplant (McCall and Shapiro, 2012).

While insulin can be used to treat T2DM, anti-diabetic medicines that improve insulin sensitivity or stimulate insulin secretion are initially recommended in combination with lifestyle intervention (Figure 1.1.7). Agents that increase insulin sensitivity include metformin and thiazolidinediones (TZDs). Metformin is currently used as the first-line treatment for newly diagnosed T2DM patients (WHO, 2009). The primary mechanism of action of metformin is to inhibit hepatic glucose production, partly through activating 5'-monophosphate-activated protein kinase (AMPK) (Bailey and Turner, 1996). TZDs are

peroxisome proliferator activated receptor γ (PPAR γ) agonists, which improve systemic insulin sensitivity and peripheral glucose uptake (Krentz et al., 2008). However, several members of the TZDs, such as rosiglitazone and pioglitazone, have received black box warnings from the FDA and its European counterpart due to an increased risk of myocardial ischemia and heart failure in some patients receiving this treatment (Dyken et al., 2007), and rosiglitazone is no longer prescribed in the UK.

Sulphonylureas, GLP-1 mimetics and dipeptidyl peptidase-4 (DPP-4) inhibitors are also prescribed to T2DM patients to improve insulin release. In addition to metformin, sulphonylureas are another first-line prescription for newly diagnosed T2DM patients due to their efficacy and long history of use to treat diabetes (WHO, 2009). They directly increase insulin secretion from β -cells by inhibiting the K_{ATP} -channel through binding to its SUR1 subunit. This results in immediate membrane depolarisation and elevated $[Ca^{2+}]_i$, facilitating insulin release (See Figure 1.1.2). Meglitinides are a class of glucose regulatory medication, which acts similarly to sulphonylureas, but with a weaker binding affinity at the SUR1 subunit and it is, therefore, ideal for offering rapid and short-lived insulin secretion that is needed after meals. GLP-1 mimetics and DPP-4 inhibitors act in a similar principle by regulating GLP-1R receptor activation in T2DM patients. As described in Section 1.1.3.4, GLP-1 enhances nutrient-induced insulin secretion and increases β -cell mass (Wang et al., 1997). Due to its short half-life of less than 2 minutes, long-lasting GLP-1 mimetics or inhibitors of DPP-4, an enzyme that degrades GLP-1, have been developed for treating T2DM patients. Clinical studies have revealed that T2DM patients treated with the GLP-1 mimetic, exenatide, have reduced on body weight compared to control individuals (Verspohl, 2009). Thus, the GLP-1 mimetics serve as attractive candidates for glycaemic control in obese T2DM patients.

Individuals unable to use the glucose-lowering medications mentioned above are advised to use α -glucosidase inhibitors, which delay intestinal carbohydrate digestion and thus delay

elevations in blood glucose levels (NICE, 2009). Moreover, A new class of anti-diabetic drug, sodium-glucose transport protein 2 (SGLT-2) inhibitors, which inhibit SGLT-2-mediated glucose re-absorption by the proximal tubule in the kidneys, was approved for clinical use in 2012. Results from randomised clinical trials have demonstrated that administration of SGLT-2 inhibitors as monotherapy or in combination with other therapies provide durable glucose-lowering efficacy in T2DM patients (Tahrani et al., 2013).

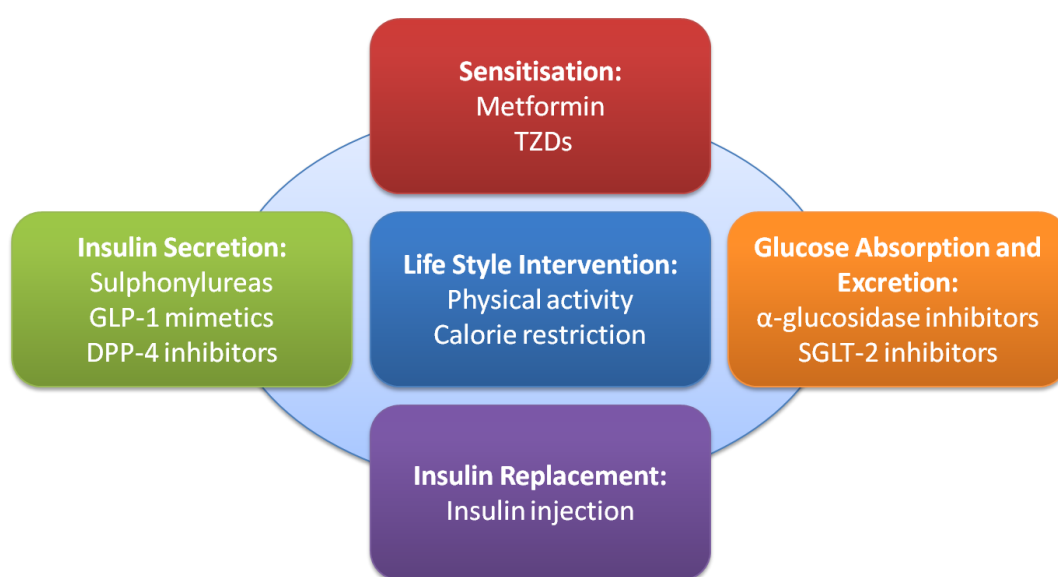


Figure 1.1.7 Therapies for T2DM.

Classes of therapies currently available for T2DM patients to maintain normal glycaemic control.

Most of the conventional therapies for diabetes listed above were developed and marketed with little understanding of defined molecular targets or underlying mechanisms of action and, therefore, have significant mechanism-based limitations and side effects. For example, hypoglycaemia is a severe side effect associated with treatment with insulin or sulphonylureas. Long-term administration of sulphonylureas and TZDs has also been reported to cause notable

weight gain (Krentz and Bailey, 2005). Moreover, there is an increased risk (almost doubled) of genito urinary infections in female patients receiving SGLT-2 treatment during the clinical trials (Tahrani et al., 2013). Therefore, new types of glucose-lowering agents are needed to offer not just complementary and additional effectiveness to existing drugs, but also benefits that are not routinely accomplished, such as reducing risks of secondary complications.

1.3 The novel cannabinoid receptor GPR55

Studies on the physiological effects of *Cannabis sativa* have identified the endocannabinoid system (ECS), which classically consists of GPCR cannabinoid receptors 1 and 2 (CB1 and CB2), the endogenous ligands 2-arachidonoyl glycerol (2-AG) and N-arachidonylethanolamine (AEA) and the enzymes responsible for their synthesis and degradation. CB1, the most abundant GPCR expressed in the brain, is a major regulator in the hypothalamic reward system controlling food intake and AEA and 2-AG trigger hyperphagia through activation of CB1 (Williams and Kirkham, 1999; Williams et al., 1998). Thus, the CB1 antagonist Rimonabant (SR141716A) was developed as an anti-obesity agent, and although it was effective in reducing food intake and body weight, it was withdrawn from clinical use after only two years due to its adverse psychological effects (Sam et al., 2011). In addition to the function of the ECS in the brain, recent research in the endocrine pancreas has revealed the expression of CB1 and CB2 in the islets of Langerhans and their coupling to second messenger generation and islet hormone secretion (Di Marzo, 2008; Li et al., 2011b). However, there is a lack of agreement on the effects of cannabinoid receptor activation on insulin secretion from islets. Thus it has been reported that pharmacological activation of CB1 and CB2 in rodent and human islets results in either stimulation (Anderson et al., 2013; Bermudez-Silva et al., 2008; Li et al., 2011a; Li et al., 2010) or inhibition (Anderson et al., 2013; Juan-Pico et al., 2006; Nakata and Yada, 2008) of insulin secretion. The reason for the inconsistent data may be because of differences in experimental design, as one recent study has shown that AEA inhibited glucose-induced insulin secretion from freshly isolated rat islets, but caused a marked increase of insulin secretion from islets cultured overnight (Anderson et al., 2013).

Research on the ECS in other cell types has demonstrated that some cannabinoid ligands that were originally thought to be specific for CB1 or CB2 can also act independently of these receptors, suggesting the existence of a third cannabinoid receptor (Curran et al., 2005; Derocq

et al., 1998; Kaplan et al., 2005; Nieri et al., 2003). GPR55 was first suggested as a novel cannabinoid receptor when *in silico* screening of patents from GlaxoSmithKline and AstraZeneca revealed that it interacts with some cannabinoid receptor agonists and antagonists (Brown, 2007). Deorphanisation of GPR55 occurred as recently as 2007, with the report that a range of endogenous and pharmacological cannabinoids activated this receptor when it was transiently expressed in human embryonic kidney 293 (HEK293) cells (Ryberg et al., 2007). Emerging evidence on the expression and signalling pathways downstream of GPR55, as described below, suggest that it may play an important role in regulating islet function and energy homeostasis.

1.3.1 GPR55 expression

Since the cloning of GPR55 in 1999 and its identification as a novel GPCR highly expressed in the human brain (Sawzdargo et al., 1999), it has been detected in various regions of the brain including the frontal cortex, striatum, hypothalamus, brain stem, hippocampus, cerebellum, caudate and putamen in rodents and humans (Baker et al., 2006; Henstridge et al., 2011; Lauckner et al., 2008; Ryberg et al., 2007; Sawzdargo et al., 1999). Intriguingly, in mouse striatum, hypothalamus and brain stem, expression levels of GPR55 mRNA are comparable to those of CB1, suggestive of an important role for GPR55 in these regions (Henstridge et al., 2011; Ryberg et al., 2007). Consistent with this, it has recently been reported that GPR55 plays a role in motor co-ordination (Wu et al., 2013).

GPR55 is also expressed in a wide range of peripheral tissues, including spleen, adrenals and bone (Ryberg et al., 2007; Sawzdargo et al., 1999; Whyte et al., 2009), and also in metabolically important cells such as adipocytes (Moreno-Navarrete et al., 2012) and the gastrointestinal tract (Lin et al., 2011; Ryberg et al., 2007; Schicho et al., 2011). In addition, GPR55 mRNA and protein have been identified in rodent β -cell lines (BR1N-BD11 and MIN6) and islets. Immunohistochemical analyses have indicated that GPR55 is expressed by β -cells

in mouse and rat islets, but it was not detected in α - or δ -cells (McKillop et al., 2013; Romero-Zerbo et al., 2011), suggesting that it might play a physiological role in regulating glucose homeostasis through effects on insulin secretion.

1.3.2 GPR55 pharmacology

GPR55 is a seven transmembrane spanning GPCR that shares only approximately 14% sequence identity with CB1 and CB2 (Baker et al., 2006) and lacks their typical ‘cannabinoid binding pocket’ (Kotsikorou et al., 2011). It is therefore rather surprising that GPR55 was considered to be a third cannabinoid receptor, and this low homology and altered cannabinoid binding site might underlie some of the controversy surrounding the interactions between cannabinoids and GPR55, as described below. Details of compounds that are reported to have significant pharmacological effects at GPR55 are described below and the key information is summarised in Table 1.3.2.1.

The abilities of AEA and 2-AG, endocannabinoids that are agonists at CB1 and CB2, to activate GPR55 were first demonstrated in GTP γ S binding assays, which indicated that AEA was equipotent in activating GPR55, CB1 and CB2, while 2-AG was up to 200-fold more potent in activating GPR55 than it was in stimulating either CB1 or CB2 (Ryberg et al., 2007). However, other experiments using GPR55-expressing HEK293 cells demonstrated that AEA, but not 2-AG, triggered GPR55-dependent increases in intracellular calcium ($[Ca^{2+}]_i$) and activation of Rho small GTPases (Lauckner et al., 2008; Ryberg et al., 2007), casting doubt on 2-AG exerting its effects via GPR55. Further evidence that AEA acts via GPR55 was provided by observations that elevations in $[Ca^{2+}]_i$ in primary human endothelial cells in response to this cannabinoid were absent following siRNA-mediated down-regulation of GPR55 (Henstridge et al., 2009b; Waldeck-Weiermair et al., 2008). Conversely, other studies using GPR55-HEK293 cells reported that AEA did not increase $[Ca^{2+}]_i$ (Oka et al., 2007; Oka et al., 2009), and it did not act as a GPR55 agonist in β -arrestin recruitment assays (Sharir and Abood, 2010), and it is

clear that AEA has promiscuous receptor selectivity with agonist actions at TRPV1 receptors (Ross, 2003) as well as at cannabinoid receptors. Thus, although several studies do support activation of GPR55 by AEA and 2-AG, there is currently no consensus between published studies on whether these cannabinoids are bona fide GPR55 agonists and their actions at other receptors makes it difficult to draw firm conclusions on their cellular mode of action.

Several studies have demonstrated that a bioactive lipid, L- α -lysophosphatidylinositol (LPI), has agonist effects in GPR55-HEK293 cells leading to suggestions that LPI is an endogenous non-cannabinoid ligand of the receptor (Henstridge et al., 2009b; Lauckner et al., 2008; Oka et al., 2007; Oka et al., 2009; Waldeck-Weiermair et al., 2008). In addition, loss of LPI-induced cellular responses following GPR55 knockdown have been observed in human endothelial cells (Kargl et al., 2013), cancer cell lines (Pineiro et al., 2011) and in rodent synaptic cells (Sylantsev et al., 2013). Despite these findings, GPR55-independent effects of LPI occurring via activation of Ca^{2+} -activated K^{+} channels have also been reported (Bondarenko et al., 2011a, b).

The phytocannabinoid cannabidiol (CBD) is a major component of cannabis and a synthetic CBD analogue, O-1602, is reported to be a potent and selective GPR55 agonist that lacks significant binding affinity for either CB1 or CB2 (Johns et al., 2007; Ryberg et al., 2007; Waldeck-Weiermair et al., 2008). O-1602 was first reported to activate a putative cannabinoid receptor distinct from CB1 and CB2 more than a decade ago (Jarai et al., 1999; Offertaler et al., 2003). It was only a relatively recent study that showed that O-1602 was able to induce activation of Rho small GPTases in GPR55-HEK293 cells (Ryberg et al., 2007). Subsequent studies in human endothelial cells have demonstrated that O-1602 treatment elevated $[\text{Ca}^{2+}]_i$ and this response was attenuated after siRNA-mediated down-regulation of GPR55 expression (Waldeck-Weiermair et al., 2008). O-1602-induced physiological effects were also observed in mouse and human osteoclasts and similar effects were not reported in osteoclasts isolated from

GPR55 knockout mice (Whyte et al., 2009). In contrast to the proposed GPR55 agonist effects of O-1602, native CBD is an effective GPR55 antagonist (Ryberg et al., 2007), which has been widely used to inhibit GPR55 agonist-induced biological effects (Lauckner et al., 2008; Ryberg et al., 2007; Whyte et al., 2009). However, a recent study has proposed that CBD may have GPR55 agonist activity since it provided protection against acute pancreatitis in mice to a similar extent to that seen using O-1602 (Yu et al., 2013). Currently there is a lack of selective GPR55 agonists and antagonists to study this receptor.

Compounds	Activity at GPR55 [ref.]	Other activities [ref.]
Anandamide (AEA)	Agonist (Henstridge et al., 2009b; Lauckner et al., 2008; Ryberg et al., 2007; Waldeck-Weiermair et al., 2008)	CB1 and CB2 agonist (Li et al., 2011b)
2-Arachidonoylglycerol (2-AG)	Agonist (Ryberg et al., 2007)	CB1 and CB2 agonist (Li et al., 2011b)
Lysophosphatidylinositol (LPI)	Agonist (Henstridge et al., 2009b; Lauckner et al., 2008; Oka et al., 2007; Oka et al., 2009; Waldeck-Weiermair et al., 2008)	Ca ²⁺ -activated K ⁺ channel agonist (Bondarenko et al., 2011a, b)
O-1602	Agonist (Johns et al., 2007; Romero-Zerbo et al., 2011; Ryberg et al., 2007; Waldeck-Weiermair et al., 2008)	---
Cannabidiol (CBD)	Antagonist (Lauckner et al., 2008; Ryberg et al., 2007; Whyte et al., 2009)	Weak CB1 and CB2 antagonist (Petitet et al., 1998)
SR141716A	Agonist (Kapur et al., 2009; Yin et al., 2009)	CB1 antagonist (Li et al., 2011b)
AM251	Agonist (Kapur et al., 2009; Yin et al., 2009)	CB1 antagonist (Li et al., 2011b)

Table 1.3.2.1 Agonist and antagonist ligands for GPR55.

The key compounds that are reported to have significant pharmacological effect at GPR55, either as agonists or antagonists, are listed in the table and information is provided on whether they have targets in addition to GPR55. More detailed information is provided in the main text.

A divergence in the effects of some ligands at CB1 and GPR55 has been observed, such that the synthetic CB1 antagonists AM251 and Rimonabant (SR141716A) have agonist effects in β -arrestin and luminescence reporter assays in cell lines engineered to express GPR55 (Kapur et al., 2009; Yin et al., 2009). Subsequent functional studies revealed that AM251 promoted GPR55-dependent Ca^{2+} mobilisation and both AM251 and SR141716A activated ERK1/2 mitogen-activated protein (MAP) kinases and phosphorylation of the transcription factors CREB and NF- κ B via GPR55 (Henstridge et al., 2010). The concentrations of these ligands that activate GPR55 are higher than those required to antagonise CB1 (Pertwee, 2005; Ryberg et al., 2007), but it is possible that Rimonabant may reach sufficient concentrations when administered *in vivo* to both inhibit CB1 and activate GPR55. The activation of GPR55 by Rimonabant, may contribute to the off-target effects associated with its therapeutic use in obesity (Kapur et al., 2009).

1.3.3 GPR55 signalling cascades

The G protein subunits with which GPR55 associates and the signalling cascades downstream of its activation have been examined in detail using GPR55-HEK293 cells. Pretreatment with pertussis toxin to ADP ribosylate Gi or with peptides to block specific Gα protein subunits indicated that GPR55 is coupled to G12/13, but not to Gs, Gi or Gq (Ryberg et al., 2007). This was confirmed in a study where overexpression of catalytically inactive G12/13 abolished LPI-induced Ca^{2+} mobilisation (Henstridge et al., 2009b). In contrast, involvement of both Gq and G12/13 was suggested by another group who reported that co-transfection of GPR55-HEK293 cells with dominant negative Gq or G12/13 reduced calcium responses to GPR55 ligands (Lauckner et al., 2008).

Experiments in cell lines and primary cells have shown that pharmacological activation of GPR55 by cannabinoids and phospholipid-derived agonists induced elevations in $[\text{Ca}^{2+}]_i$ (Henstridge et al., 2010; Kargl et al., 2013; Oka et al., 2007; Pineiro et al., 2011; Sylantyev et al., 2013; Waldeck-Weiermair et al., 2008; Yu et al., 2013), and these responses were a consequence of Ca^{2+} release from the ER following activation of PLC and IP_3 generation (Lauckner et al., 2008; Waldeck-Weiermair et al., 2008). Both Gq and G12/13 were found to be involved in GPR55-mediated PLC activation (Lauckner et al., 2008). In addition, Rho small GTPases, including RhoA, Cdc42 and Rac1, are another class of second messengers that are considered to be activated by GPR55 via G12/13 (Obara et al., 2011; Ryberg et al., 2007; Whyte et al., 2009). These small GTPases in turn activate ROCK, which can increase $[\text{Ca}^{2+}]_i$ both via PLC-mediated PIP_2 hydrolysis and IP_3 generation, and through actin cytoskeleton remodelling (Lauckner et al., 2008).

Activation of GPR55 also leads to phosphorylation of the MAP kinases ERK1/2 via the RhoA-ROCK pathway and/or increases in $[\text{Ca}^{2+}]_i$ (Anavi-Goffer et al., 2012; Henstridge et al., 2009b; Oka et al., 2010; Pineiro et al., 2011; Waldeck-Weiermair et al., 2008). The transcription

factors NF- κ B and CREB, which are downstream of the ERK pathway, are activated by GPR55 (Henstridge et al., 2010), as is activating transcription factor 2 (ATF-2), although this occurs via p38 MAPK, not through ERK1/2 (Oka et al., 2010). In addition, GPR55 can also activate the transcription factor nuclear factor of activated T-cells (NFAT) in a RhoA-dependent manner (Henstridge et al., 2009b). Once activated, these transcription factors regulate expression of key genes that are responsible for transducing the effects of GPR55 in a diverse range of cellular functions including tumorigenesis, bone resorption and neuropathic pain (Henstridge, 2012). It is interesting to note that some of the GPR55-mediated signalling is ligand-specific. For instance, AM251 and SR141716A, but not LPI, were able to induce CREB phosphorylation (Henstridge et al., 2010). It is clear that further research is needed to fully elucidate the GPR55 signal transduction cascades, but our current understanding of the key pathways through which this receptor is reported to act are summarised in Figure 1.1.8.

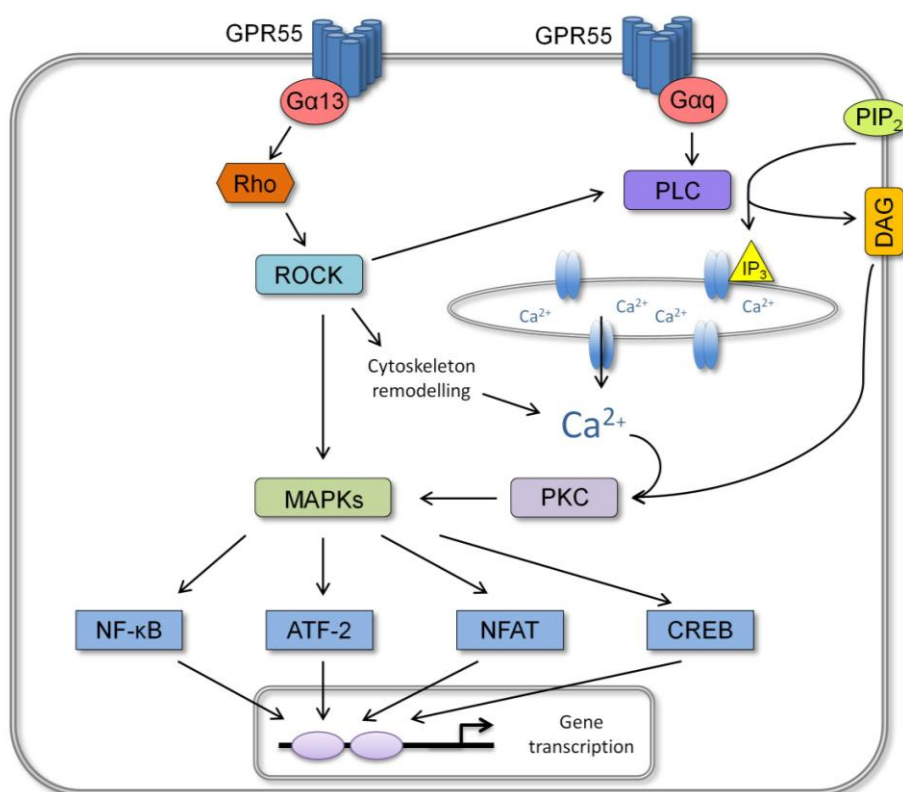


Figure 1.1.8 Schematic representation of signalling pathways downstream of GPR55 activation.

The diagram shows the main pathways through which GPR55 signals to exert its effects on cellular function. It is widely accepted to be coupled to the G12/13 subunit, which activates Rho small GTPases and their downstream effector ROCK. Activated ROCK can elevate intracellular Ca²⁺ either through PLC-induced PIP₂ hydrolysis and IP₃-mediated Ca²⁺ mobilisation from the ER or cytoskeleton remodelling. In addition, ROCK can modulate a range of gene transcription factors including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), activating transcription factor 2 (ATF-2), nuclear factor of activated T-cells (NFAT) and cAMP response element-binding protein (CREB), through activation of mitogen-activated protein kinases (MAPKs) such as ERK1/2 and p38 MAPK. GPR55 can also couple to Gq, which increases intracellular Ca²⁺ through PLC activation. Ca²⁺ and DAG activate PKC, which also regulates transcription factor activity through MAPKs.

1.3.4 Regulation of islet functions by GPR55

During the course of this thesis, two studies were published that reported GPR55 expression in rodent β -cells and islets (McKillop et al., 2013; Romero-Zerbo et al., 2011), suggesting that it might play a physiological role in regulating glucose homeostasis through effects on insulin secretion. Indeed, O-1602 potentiated glucose-stimulated insulin secretion from mouse and rat islets, indicative of a direct stimulatory role for GPR55 in β -cells (McKillop et al., 2013; Romero-Zerbo et al., 2011). In the recent report evaluating the effects of various GPR55 ligands on insulin secretion it was found that all agonists used -AM251, O-1602, abnormal CBD (a synthetic GPR55-activating CBD analogue) and the phospholipid-derived endocannabinoids oleoylethanolamine (OEA) and phenethylamine (PEA) - stimulated insulin secretion from insulin-secreting cell line BR1N-BD11 cells and mouse islets, and their effects were inhibited by CBD (McKillop et al., 2013), suggesting that they acted via GPR55 activation. *In vivo* studies have also confirmed that short-term administration of GPR55 agonists to rats or mice increased plasma insulin levels and improved glucose tolerance (McKillop et al., 2013; Romero-Zerbo et al., 2011). These observations imply that GPR55 agonists have acute, beneficial effects on insulin secretion and glucose handling.

LPI has long been known to be an insulin secretagogue (Metz, 1986), and although LPI-induced insulin secretion was suggested to be at least partly mediated by the mobilisation of $[Ca^{2+}]_i$ in β -cells (Metz, 1988), its precise mode of action in islets has never been established. The identification of LPI as an endogenous ligand of GPR55 (Oka et al., 2007) points to a possibility that the insulintropic effect of LPI could be mediated through activation of this receptor, but there is no published data that has addressed this hypothesis.

There has been some investigation of the signalling cascades downstream of GPR55 activation in β -cells that suggest that elevations in $[Ca^{2+}]_i$ are important in transducing the stimulatory effects of GPR55 agonists. Thus, O-1602 induced transient increases in $[Ca^{2+}]_i$ in rat islets

(Romero-Zerbo et al., 2011), and AM251, O-1602, abnormal CBD, OEA and PEA all caused rapid increases in $[Ca^{2+}]_i$ in insulin-secreting MIN and BR1N-BD11 cells (McKillop et al., 2013; Ning et al., 2008), consistent with GPR55 signalling via PLC activation in β -cells (Figure 1.1.8). These agonists also stimulated small increases in β -cell intracellular cAMP levels (McKillop et al., 2013; Ning et al., 2008), which are likely to be secondary to activation of calcium-sensitive isoforms of ACs since there is no evidence that GPR55 is directly coupled to Gs to promote cAMP accumulation.

GPR55-mediated activation of PLC can occur both via conventional Gq coupling and also by the G12/13 pathway (Figure 1.1.8) so it is possible that GPR55 also regulates β -cells via G12/13 signalling since this G protein has previously been identified in islets (Hammar et al., 2009; Skoglund et al., 1999). However, inhibition of the Rho kinase ROCK causes increased insulin release from FACS-purified rat β -cells (Hammar et al., 2009) and this negative role for RhoA on insulin release is inconsistent with the observed stimulatory effects of GPR55 agonists summarised above. It is therefore possible that there are both stimulatory and inhibitory cascades activated by GPR55 agonists in islets, via separate G proteins and further work is required to define the signalling cascades that regulate insulin exocytosis downstream of GPR55 activation.

GPR55 may also play a role in maintaining functional insulin-producing β -cells, since OEA can protect β -cells from palmitate-induced apoptosis, an effect that was not mediated via another OEA receptor, GPR119 (Stone et al., 2012). O-1602 was reported to promote phosphorylation of ERK1/2 and CREB in various cell types (Andradas et al., 2011; Henstridge et al., 2010; Pineiro et al., 2011; Whyte et al., 2009) (Figure 1.1.8), effectors that are known to regulate β -cell mass (Burns et al., 2000; Jhala et al., 2003). Two studies in cancer cell lines demonstrated that activation of GPR55 promotes cell proliferation through PI3K/Akt or MAPK pathways (Andradas et al., 2011; Pineiro et al., 2011). However, there has been no

published studies investigating whether activation of GPR55 in β -cells contributes to maintenance of β -cell mass.

1.3.5 GPR55 knockout mice

GPR55 knockout (KO) mice were first generated and backcrossed into the C57/BL6 mouse strain by a research group at the University of Aberdeen (Whyte et al., 2009). A GPR55 KO mouse colony was established at the Guy's Campus of King's College London in accordance with UK Home Office standards in 2012, and these mice were used in some of the experiments described in this thesis. General phenotypic analysis of GPR55 KO mice revealed no obvious primary differences to their wildtype littermates. However, they were reported to have increased bone mass (Whyte et al., 2009) and impaired coordination towards tasks requiring challenging motor responses (Wu et al., 2013), suggesting that GPR55 plays a role in bone metabolism and motor coordination. Another study using these mice has shown that GPR55 KO mice failed to develop mechanical hyperalgesia against inflammatory and neuropathic pain (Staton et al., 2008). In our lab, we have also observed that GPR55 KO mice have a larger rib cage, possibly due to the suggested regulatory role of GPR55 in bone metabolism (Whyte et al., 2009). In terms of metabolism, preliminary data generated from an *in vivo* project on GPR55 in our lab, separate from the studies described in this thesis, have shown that GPR55 KO mice had normal glucose tolerance when fed on a normal chow diet, but they were more susceptible to diet-induced obesity and glucose intolerance than wildtype mice when they were fed on a high fat diet (Liu and Persaud, unpublished). These observations strongly suggest that GPR55 plays a role in maintaining glucose homeostasis, at least under conditions of metabolic stress, and the *in vitro* investigations described in this thesis will provide further insights into the role of this receptor in islets.

1.4 Aims

The principal objective of the experiments described in this thesis was to determine the expression and functional roles of GPR55 in islets of Langerhans, and identify the signalling mechanisms that are involved in the processes by which GPR55 ligands regulate insulin secretion. Experiments detailed in this thesis will use the pharmacological GPR55 agonist O-1602, the putative endogenous ligand LPI and the GPR55 antagonist CBD to manipulate GPR55 activity in islets. Islets isolated from GPR55 KO mice will also be investigated in an attempt to understand whether these ligands act specifically on GPR55 to regulate islet functions. In addition, due to the lack of knowledge of GPR55 in human islets, this thesis will also investigate the expression and functional roles of GPR55 for the first time in isolated human islets. An outline of the aims of this thesis is given below.

- To determine the expression of GPR55 in MIN6 β -cells, and in mouse and human islets.
- To investigate the role of GPR55 activity and the specificity of GPR55 ligands in regulating $[Ca^{2+}]_i$ and RhoA activity in MIN6 cells, and when possible, in isolated mouse and human islets.
- To examine the effects and specificity of GPR55 ligands on insulin secretion from isolated mouse and human islets.
- To investigate the effects of pharmacological manipulations of GPR55 and GPR55 deletion on mouse islet apoptosis.

Chapter 2 Methods

CHAPTER 2 METHODS

2.1 Materials

Tissue culture flasks, Petri dishes and cover slips were supplied by Thermo Fisher Scientific (Leicestershire, UK). L- α -lysophosphatidylinositol sodium salt from *Glycine max*, cell dissociation solution, cell culture media, agarose gel and all other chemicals and reagents were purchased from Sigma-Aldrich (Dorset, UK). Protease inhibitor cocktail (X100) reagent was bought from Calbiochem (Nottingham, UK). 10% pre-made polyacrylamide gels, NuPAGE® sample buffer and transfer buffer were purchased from Invitrogen (Paisley, UK). RNA isolation kits, PCR reaction reagents and DNA purification kits were purchased from Qiagen (Cambridge, UK). ECL western blotting reagents and Hyperfilm were from GE Healthcare (Little Chalfont, UK). The rabbit anti-GPR55 antibody was obtained from Cayman Chemical Company (Cambridge, UK) and anti-rabbit secondary antibody was bought from Thermo Fisher Scientific (Leicestershire, UK). Rainbow protein molecular weight markers and polyvinylidene fluoride (PVDF) membrane were bought from Millipore (Watford, UK). Cell-Tak™ cell and tissue adhesive was purchased from BD Bioscience (Oxford, UK). CBD and O-1602 (5-methyl-4[(1R,6R)-3-methyl-6-(1-cyclohexen-1-yl)]-1,3-benzenediol) were supplied by Tocris Biosciences (Bristol, UK). The RhoA activation assay kit was purchased from Cytoskeleton Inc. (Denver, USA).

2.2 Cell Culture

2.2.1 Mouse insulinoma (MIN6) β -cells

Although β -cells are the primary cell type in islets, the presence of other endocrine and non-endocrine cells can lead to difficulties in defining β -cell-specific roles when using whole islets. For this reason, clonal insulin-secreting cell lines are often used experimentally as a readily available source of pure β -cells (Persaud, 1999). Mouse insulinoma (MIN6) β -cells preferentially express GLUT-2 and glucokinase for glucose transport and phosphorylation

respectively. They also have a relatively high insulin content (~20% of an average mouse β -cell) in comparison to other insulin-secreting lines such as RIN-m5F cells (Persaud, 1999). These functional similarities to primary islet β -cells, together with their rapid growth characteristics, made them the β -cell line of choice for some of the experiments described in this thesis.

2.2.2 Maintenance of MIN6 cells

MIN6 cells of passage 28-40 were cultured as monolayers on negatively charged tissue culture plastic and were maintained in culture in Dulbecco's Modified Eagle Medium (DMEM) containing 25mM glucose supplemented with 10% foetal bovine serum (FBS), 2mM L-glutamine and 100U/ml penicillin/100 μ g/ml streptomycin. Cells were maintained at 37°C in an atmosphere of 95% air/5% CO₂ and the growth medium was replaced every 3-4 days.

2.2.3 Subculturing

MIN6 cells were subcultured by trypsinisation to maintain them at a logarithmic growth rate when they reached approximately 70% confluency. The growth medium was removed and cells were rinsed with phosphate buffered saline (PBS) before being exposed to trypsin/EDTA (0.1%/0.02%) for 3 minutes at 37°C. Trypsin helps to remove cells by enzymatically cleaving the adhesion molecules between the cells and the tissue culture surface and the EDTA chelates Ca²⁺ which is required for Ca²⁺-dependent adhesion. Activity of trypsin was inhibited by adding medium supplemented with FBS and the suspended MIN6 cells were subcultured in fresh culture flasks.

2.2.4 Estimation of cell number

A Neubauer haemocytometer is used when an estimation of cell number is required. Following trypsinisation, the cells were re-suspended in 1ml growth medium and a 10 μ l aliquot of the

cell suspension was added to 90µl PBS (1:10 dilution), of which 10µl was loaded onto the haemocytometer (Figure 2.1.1) is a thick glass microscope slide with a grid of perpendicular grooves. By placing a cover-slip on top of it, chambers of 0.1mm in depth and sides of 1mm in diameter are formed. A diluted MIN6 cell suspension was loaded against the cover-slip, and was dispersed into the chambers due to capillary action. The number of cells was counted in each of the four 1x1mm corner squares under a light microscope. Total cell number per ml was estimated using the following calculation:

$$\text{Cell number per ml} = \text{average cell number per } 0.1\text{mm}^2 \times \text{dilution factor}$$

$$(\text{e.g. } 10 \text{ for } 1 \text{ in } 10 \text{ dilution}) \times 10^4$$

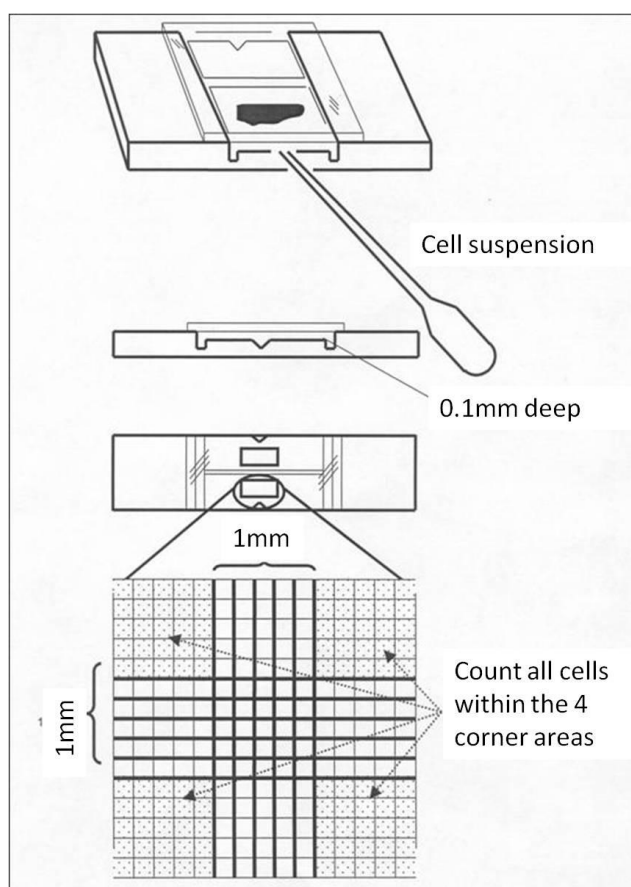


Figure 2.1.1 Cell number estimation with a Neubauer haemocytometer.

The haemocytometer is engraved with grids of perpendicular lines to make up 9 large squares. Each square is 1mm² with a depth of 0.1mm below the cover-slip giving a total volume of 0.1mm³ per square. By counting the number of cells in each square, the total number of cells in the original cell suspension can be calculated. Figure adapted from:

(http://www.who.int/vaccines/en/poliolab/webhelp/Chapter_04/4_2_Cell_culture_procedures.htm)

2.2.5 Freezing down and thawing of cells

In order to preserve MIN6 cells for future use, they were trypsinised, pelleted and re-suspended in cryopreservant (90% FCS/10% dimethyl sulfoxide (DMSO), v/v). Cryo-safe tubes containing 1ml aliquots of cell suspension were stored at -80°C in isopropanol to allow a gradual freezing process before being stored in liquid nitrogen (-196°C).

To retrieve frozen cells, cell suspensions were rapidly thawed in a 37°C water bath and transferred to a 15ml centrifuge tube. 10ml pre-warmed growth medium was added and cells were pelleted and resuspended in fresh growth medium. This process removed DMSO in the cryopreservant, which is detrimental to cell growth. The cell suspension was then transferred into a fresh tissue culture flask.

2.3 Islet isolation

2.3.1 Isolation of human islets of Langerhans

Human pancreata were removed from healthy, hearting-beating cadaver organ donors and human islets were isolated from these pancreata under aseptic conditions at the King's College Human Islet Isolation Unit by Dr. Guocai Huang. Islets were then maintained at 37°C (95% air, 5% CO₂) in Connaught Medical Research Laboratories (CMRL) culture medium containing 5.6mM glucose and supplemented with 10% foetal bovine serum (FBS), 2mM L-glutamine and 100U/ml penicillin / 100µg/ml streptomycin. Human islets were kept at Human Islet Isolation Unit for 12-24 hours before transferring to Guy's Campus and they were used within 48 hours after arrival. Ethical approval for the use of human islets for research was given by King's College Hospital Ethics Committee and agreement for using human material for research was reached with donor relatives.

2.3.2 Isolation of mouse islets of Langerhans

Mouse islets were isolated from either ICR, WT C57/BL6 mice and GPR55 KO C57/BL6 mice (described in Section 1.3.5) after collagenase digestion of the pancreas. The duct connecting the pancreas with the intestines was first clamped before injection of collagenase into the pancreas via the bile duct. Approximately 2ml of 1mg/ml collagenase solution was injected using a 2mm needle and the inflated pancreas was removed from the mouse and transferred to a 50ml centrifuge tube on ice. The collected pancreas tissues were incubated in 37°C water bath for 10 minutes to allow collagenase digestion of exocrine tissues. Digestion was stopped by transferring tubes to ice and tissues were washed with ice cold Minimum Essential Medium (MEM) (supplemented with 10% FBS and 100U/ml penicillin/100µg/ml streptomycin) before filtering through a 2µm sieve to remove undigested exocrine tissue and other tissues such as fat. Digested pancreas tissues were re-suspended in a purification gradient Histopaque[®] solution, containing 1.077g/ml polysucrose. MEM was gently added on top of the Histopaque[®] solution before centrifugation at 11,000 rpm and islets were retrieved from the interface between the Histopaque[®] solution and MEM. Islets were further washed in aseptic conditions to remove residual Histopaque[®] solution and potential contaminants. Islets were cultured in RPMI-1640 medium containing 11mM glucose supplemented with 10% FBS, 2mM L-glutamine and 100U/ml penicillin/100µg/ml streptomycin, and maintained at 37°C (95% air, 5% CO₂) for up to 2 days before experimental use.

2.4 Gene expression

2.4.1 RNA extraction

In order to investigate expression of particular genes by eukaryotic cells, a complementary DNA (cDNA) library containing the coding regions is prepared from total cellular messenger RNA (mRNA). In this thesis, total RNA was isolated from MIN6 cells or islets and mRNA was separated from other RNAs such as ribosomal RNA (rRNA) and transfer RNA (tRNA) using the RNeasy Mini kit from Qiagen, according to the manufacturer's instructions.

MIN6 cells (P28-40) grown as monolayers in T75 flasks (30-50% confluency) or approximately 100 islets were harvested and re-suspended in 350µl RLT Buffer (provided with the RNeasy kit) supplemented with 0.1% β-mercaptoethanol in a 1.5ml Eppendorf tube. MIN6 cells were lysed by vortexing and islets were passed through an RNeasy Shredder Column for homogenisation. 350µl of 70% ethanol was added to lysed samples before being transferred onto an RNeasy Mini spin column placed in a 2ml collection tube. The mixture was spun for 15 seconds at 12,000 rpm and the flow-through was discarded. The spin column was washed with 350µl RW1 Buffer before the mixture of 10µl DNase I stock solution and 70µl RDD Buffer was directly pipetted on top of the spin column membrane, with incubation for 15 minutes at room temperature. The DNase solution was washed off by adding 350µl of RW1 Buffer and centrifugation at 12,000 rpm for 15 seconds. The spin column membrane was further washed twice with 500µl RPE Buffer and total RNA was eluted through the spin column membrane in 20-40µl of RNase-free water (12,000 rpm for 1 minute).

Concentrations of isolated RNA were quantified using the ND1000 spectrophotometer (NanoDrop). 1µl of each RNA sample was loaded onto the aperture and absorbances at 260nm and 280nm were measured from the samples. The NanoDrop software applies the Beer-Lambert law, which predicts a linear change in absorbance in a solution, to determine RNA concentration, and the ratio of $A_{260\text{nm}}/A_{280\text{nm}}$ was used to assess the quality of RNA samples. A ratio of <1.6 indicates contamination of samples with proteins, phenol or other compounds that absorb 280nm emission. RNA samples with $A_{260\text{nm}}/A_{280\text{nm}} > 1.6$ were stored at -80°C and used for cDNA synthesis (Section 2.3.2).

2.4.2 cDNA synthesis

In order to amplify RNA expression using PCR, isolated single-stranded mRNA is transcribed into complementary DNA (cDNA) by an RNA-dependent DNA polymerase (reverse transcriptase). Isolated mRNA was first diluted with DNase/RNase-free water to give

approximately 50ng/ μ l, and 10 μ l of diluted mRNA was added to a DNase/RNase-free Eppendorf tube. 0.5 μ l of 10-meroligonucleotide (2 μ g/ μ l stock) and 0.5 μ l oligo-(dT)₁₈ (2 μ g/ μ l stock) were added to the same tube. The mixtures were vortexed and heated up to 70°C for 5 minutes to denature mRNA secondary structure. Samples were then placed on ice to avoid possible heat-inactivation of MMLV-Reverse Transcriptase (MMLV-RT) in the following procedure: 9 μ l of Master Mix containing MMLV-RT was prepared as shown in Table 2.3.2.1 and added to 11 μ l of the RNA/oligo mixture. Reverse transcription was then carried out using a thermal cycler (50 minutes incubation at 42°C followed by 15 minutes incubation at 72°C), cDNA products were then stored at -20°C and used for RT-PCR amplifications (Section 2.3.3).

Master Mix (stock concentration)	For 1 sample	Final Concentration (in 20 μ l)
5X RT buffer (Promega MMLV-RT kit)	4 μ l	--
DNase/RNase-free H ₂ O	0.5 μ l	--
DTT (400mM)	0.5 μ l	10mM
RNasin (40U/ μ l)	2 μ l	4U/ μ l
dNTPs (10mM)	1 μ l	500 μ M
MMLV-RT (200U/ μ l)	1 μ l	10U/ μ l

Table 2.3.2.1 Master Mix for Reverse Transcription.

2.4.3 Reverse-transcriptase polymerase chain reaction (RT-PCR)

PCR is a technique used to amplify specific regions of interest from template cDNAs *in vitro*. Pairs of sequences designed to bind complementarily to two ends of the region of interest are known as primers, which are essential for DNA polymerase to replicate DNA for amplification (Figure 2.3.3.1). In brief, PCR amplifications are carried out in a thermal cycler in repeated cycles of three phases: denaturing of double stranded DNAs at 95°C, annealing between primers and DNA templates at ~50-60°C, and extension of newly amplified DNAs of interest at 72°C. The annealing temperatures are usually determined by the percentage of guanine and cytosine nucleotides in the DNA sequence of interest and by primer length.

$$\text{Annealing temperature} = 61.2\% \pm 0.41 \times (\%GC \text{ pairs}) - (500/\text{primer length})$$

In this PhD thesis, cDNAs from MIN6 cells, mouse islets and human islets were analysed by PCR amplifications. Specific pairs of primers were designed using Primer3web and ordered from Integrated DNA Technology to amplify GPR55 mRNA. Primers were designed to be intron-spanning to avoid co-amplification of the same amplicon size from any contaminating genomic DNA, and their GC content was 55% for an optimum annealing temperature allowing high PCR yield and specificity. Annealing temperatures were further optimised after running the reactions under temperature gradient $\pm 3^\circ\text{C}$ from the predicted annealing temperature according to the formula above.

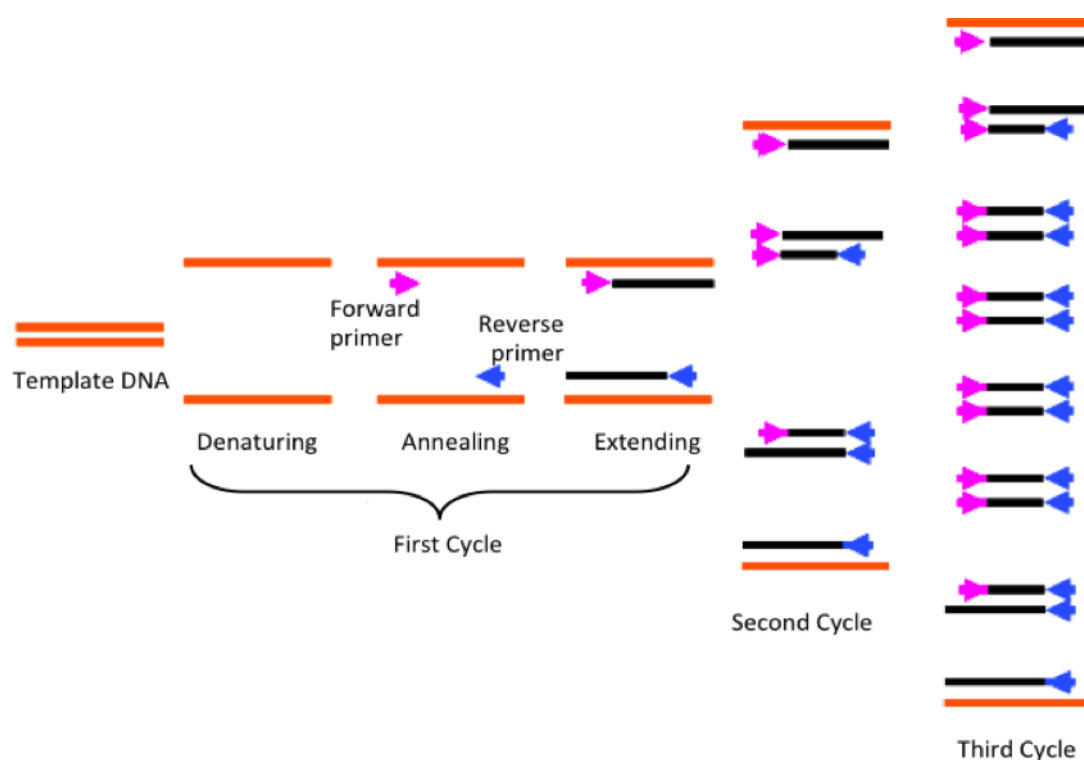


Figure 2.3.3.1 PCR amplification.

Template DNA is denatured at 95°C to give single-stranded DNA. The reaction is then cooled to ~50-60°C to allow annealing of primers onto template strands. DNA polymerase then recognises the 5' end of primers and extends DNA in a 5' to 3' direction to produce a new complementary DNA. This cycle is repeated 30-40 times. Figure adapted from:

www.obgynacademy.com/basicsciences/fetology/genetics/.

PCR master mixes of total 19µl containing Promega Go-Taq® Flexi DNA Polymerase, MgCl₂ and specific primers were prepared in DNase/RNase-free Eppendorf tubes according to Table 2.3.3.1. 1µl of target cDNA, or H₂O as a negative control, was added to the PCR master mixture, which was then vortexed and loaded onto a thermal cycler. PCR reactions proceeded as described above using primer sequences, annealing temperature, number of PCR cycles and predicted product size as listed in Table 2.3.3.2.

After the reactions were complete, 8µl of PCR products were loaded on a 1.8% agarose gel containing 5µg/ml ethidium bromide and separated by electrophoresis (70V, 40 minutes). The

gel was visualised under UV light after electrophoresis. The size of each product was compared against a Promega 100bp DNA ladder containing 100bp fragments of DNAs between 100-1,000bp. The size of each product was then compared against the predicted size from *in silico* design.

Master Mix (stock concentration)	For 1 sample	Final Concentration (in 20µl)
5X GoTaq [®] Flexi Buffer	4µl	--
DNase/RNase-free H ₂ O	10.8µl	--
cDNA or DNase/RNase-free H ₂ O	1µl	--
Forward primer (100µM)	1µl	5µM
Reverse primer (100µM)	1µl	5µM
GoTaq DNA Polymerase (5U/µl)	0.2µl	0.05U/µl
dNTPs (10mM)	0.8µl	0.4mM
MgCl ₂ (25mM)	1.2µl	1.5mM

Table 2.3.3.1 Master Mix for RT-PCR.

Gene Name	Primer Sequence	Annealing Temp.	No. of PCR Cycles	Product Size
GPR55 Receptor (mouse)	F: AGCCTTCTGACTTGGACAGC R: CCTCATCCCCTTCATACTGG	60°C	38	60bp
GPR55 Receptor (human)	F: CTGCCTTGGTTCCACCATA R: CCAGGATGCAGGTGAGTAAGA	60°C	38	60bp

Table 2.3.3.2 PCR product information.

2.5 Protein expression

2.5.1 Protein extraction

MIN6 cells were maintained as monolayers in 10cm Petri dishes up to 75% confluency and then harvested by trypsinisation and washed with PBS. Mouse and human islets were maintained in culture, pelleted and washed with PBS. Cell lysis buffer supplemented with phosphatase and protease inhibitors (Table 2.4.1.1) was added to pelleted MIN6 cells and islets, followed by brief sonication on ice to facilitate lysis (15s, 4~6μ). Samples were centrifuged at 10,000 rpm for 1 minute at 4°C and supernatants were transferred to fresh 0.5ml pre-chilled Eppendorf tubes. 5-10μl of samples were used for protein content quantification (Section 2.4.2) and the remaining samples were kept at -20°C in 4x NuPAGE® sample buffer containing denaturing agents, prior to electrophoresis on polyacrylamide gels (Section 2.4.3).

Material	Amount	Enzyme Name	Final Concentration
RIPA buffer (Sigma)	10ml	--	--
Phosphatase inhibitors tablet (Roche PhosSTOP tablet)	1 tablet	Calf Alkaline Phosphatase	14U/ml
		Potato Acidic Phosphatase	0.2U/ml
		Human Acidic Phosphatase	64U/ml
		Rabbit PP1	20U/ml
		Human PP2A	50U/ml
		Human PTP	50U/ml
Protease inhibitors tablet (Roche cOmplete, ULTRA tablet)	1 tablet	Pancreas-extract	27μg/ml
		Trypsin	3.6μg/ml
		Papain	3μg/ml
		Proteinase K	0.8μg/ml

Table 2.4.1.1 Cell lysis buffer.

Phosphatase inhibitors and protease inhibitors tablets were added to 10ml RIPA buffer and kept on ice to allow complete dissolution. Prepared cell lysis buffer can be stored at 4°C for up to 4 weeks.

2.5.2 BCA protein content quantification assay

Thermo Scientific bicinchoninic acid (BCA) protein assay reagent was used to determine protein content of samples obtained after protein extraction. In brief, protein in an alkaline environment will reduce Cu^{2+} to Cu^{1+} and BCA will react with the reduced cation to form the BCA/ Cu^{1+} complex. The complex is water-soluble and exhibits a strong linear absorbance at 550-570nm with increasing protein concentrations.

Protein samples were assayed at dilutions of 1 in 10 and 1 in 100 in cell lysis buffer, together with a series of reference standards (0, 0.03125, 0.0625, 0.125, 0.25, 0.5, 1 and 2mg/ml) prepared by serial dilution of 2mg/ml BSA in cell lysis buffer. 25 μ l of standards and samples were added to designated wells in triplicate on a 96-well plate. 200 μ l of BCA working solution (BCA solution A to BCA solution B: 50:1) was added to each well and solutions were mixed by placing on a plate shaker for 30 seconds. The plate was then incubated at 37°C for 30 minutes before reading on a Chameleon plate reader on an absorbance setting of 562nm. Sample protein concentrations were estimated based on a standard curve of absorbance versus protein concentration (Figure 2.4.1.1).

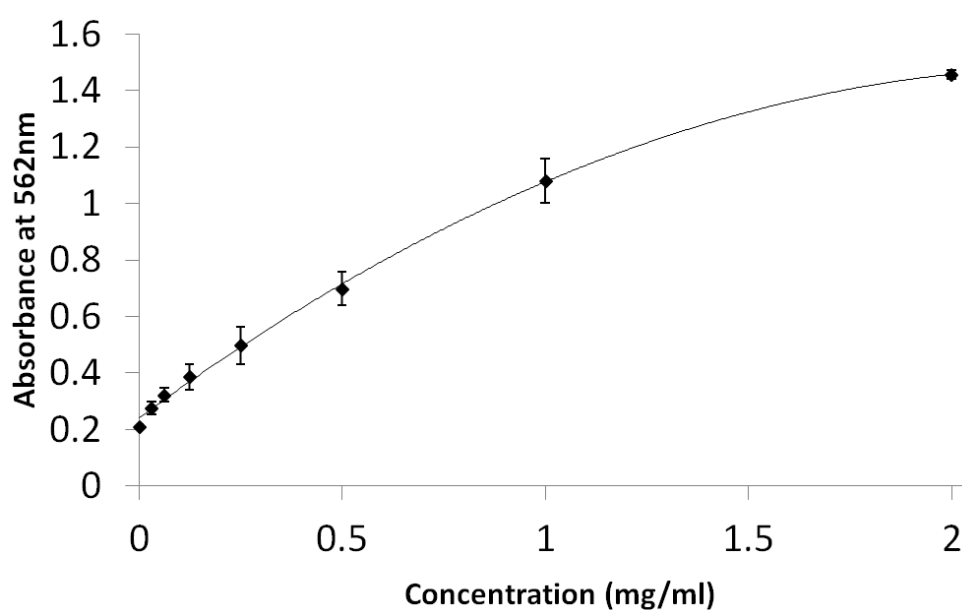


Figure 2.4.1.1 Typical standard curve for the BCA protein assay.

The absorbance of each set of standards (in triplicate) is measured at 562nm using a plate reader. A standard curve is then generated by plotting the average absorbance against the corresponding protein concentrations.

2.5.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is carried out to separate proteins according to their molecular weights across an electric field. Sodium dodecyl sulphate (SDS) is an anionic detergent that will unfold proteins into their primary structures and apply them with a negative charge. When an electric field is applied, negatively charged proteins migrate towards the cation pole through a porous gel matrix of polyacrylamide. The percentage of polyacrylamide determines the efficiency of protein mobility resulting in a fractionation according to their mass-to-charge ratio. The Life Sciences Technology NuPAGE Bis-Tris electrophoresis system was used here. Pre-determined amounts of proteins were loaded onto a pre-made 10% Bis-Tris-HCl buffered (pH 6.4) polyacrylamide gel. The gel was then secured in the NuPAGE mini cell apparatus and MOPS running buffer containing antioxidant was added to the inner chamber (Table 2.4.3.1). The outer chamber was filled with approximately 600ml MOPS running buffer before the gel was run at 200V for 70 minutes. A Rainbow[®] coloured protein molecular weight marker containing a mixture of coloured proteins of a variety of sizes was loaded and run in parallel to the sample protein extracts for estimating sample protein molecular weights.

Reagent	Molecular Weight	Amount for 500ml	Final Concentration
MOPS	209.3 g/mol	104.6g	1M
Tris Base	121.1 g/mol	66.6g	1M
SDS	288.4 g/mol	10g	69.3mM
EDTA	292.2g/mol	3g	20.5mM

Table 2.4.3.1 Preparation of 20x MOPS running buffer.

Reagents listed above were first dissolved in 400ml DI H₂O and adjusted to a final volume of 500ml. the 20xMOPS running buffer is kept at 4°C and diluted 1 in 20 with DI H₂O before use.

2.5.4 Western blotting

After SDS-PAGE, the separated proteins are transferred onto a polyvinylidene difluoride (PVDF) membrane to allow blotting with suitable antibodies. The transfer procedure is carried out by technique known as Western blotting in which the PVDF membrane and polyacrylamide gel are placed in between two layers of transfer buffer-soaked (Table 2.4.4.1) filter paper and an electric current is applied to pull proteins from the gel onto the PVDF membrane (Figure 2.4.4.1).

After transferring the proteins, non-specific binding sites on the membrane were blocked by incubating it in a protein-rich blocking buffer (5% skimmed milk powder dissolved in Tris-buffered Saline (TBS)-Tween) for 1 hour at room temperature. After this, the protein of interest was immunoblotted by incubating the membrane (16 hours, 4°C) in blocking buffer containing primary antibody raised against the target protein (1:160 dilution for the anti-GPR55 primary antibody).

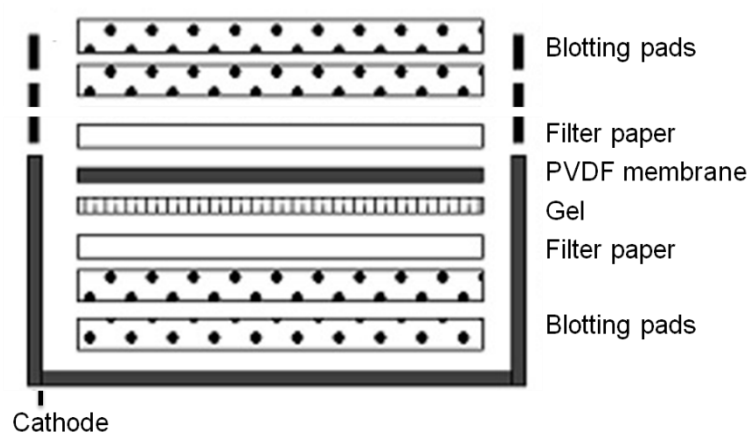


Figure 2.4.4.1 A diagrammatic representation of the polyacrylamide gel/PVDF membrane blotting module assembly.

The PVDF membrane is placed on top of the gel and both are sandwiched between filter papers and blotting pads, pre-soaked in transfer buffer. The gel/membrane assembly is placed in the module such that the gel is closest to the cathode plate. When an electric current is applied, proteins are transferred from the gel to the PVDF membrane.

After primary antibody incubation, the membrane was washed with TBS-Tween buffer and blocking buffer to remove unbound primary antibody. The membrane was further incubated for 1 hour in blocking buffer supplemented with horseradish peroxidase-conjugated secondary antibody (1:7,500 dilution for anti-rabbit secondary antibody) at room temperature. The membrane was washed again with TBS-Tween buffer before detecting immunoreactive proteins using a chemiluminescent detection method following addition of enhanced chemiluminescent (ECL) reagents. Light signals emitted by the secondary antibody were captured by photographic film and detected immunoreactive proteins were compared against migration of the Rainbow[®] markers to determine their molecular weights.

Reagent	Volume
DI H₂O	849ml
20x NuPAGE transfer buffer	50ml
NuPAGE sample antioxidant	1ml
Methanol	100ml

Table 2.4.4.1 Preparation of 1x Transfer Buffer.

Reagents listed above were mixed together to give 1l of 1x Transfer Buffer for Western blotting. 1x Transfer Buffer is stored at room temperature.

2.6 Measurement of second messenger generation

2.6.1 Calcium microfluorimetry

The single cell calcium microfluorimetry technique was used in this thesis to investigate changes in $[Ca^{2+}]_i$ in MIN6 cells and mouse and human islet cells. Fura-2 AM, a fluorescent dye, was used to measure change in intracellular calcium concentrations since its peak excitation wavelength changes from 380nm to 340nm after binding to divalent cations. Emitted fluorescent signals from Fura-2 AM were recorded at 510nm and the ratio of changes of emission intensity were used to indicate changes in $[Ca^{2+}]_i$.

Approximately trypsinised 30,000 MIN6 cells in 100µl of serum-free DMEM were seeded onto 2.2cm circular acid/ethanol-washed sterile glass cover-slips. 50-80 mouse or human islets were first dissociated using Cell Dissociation Solution before being resuspended in 40µl serum-free RPMI and seeded onto Cell-Tak[®]-coated cover-slips (prepared as in Table 2.6.1.1). Each cover-slip with cells were incubated in culture in one well of a 6-well plate at 37°C to allow cell adhesion. After 2 hours incubation, 2ml of DMEM (MIN6) or RPMI (islets) supplemented with 10% FBS was added to each well and cells were then cultured overnight. In the next day, the cells were incubated in DMEM or RPMI containing 5µM Fura-2 AM for at least 30 minutes before the experiments. The cover-slip with Fura-2-loaded cells was placed onto a 37°C-heated stage of a fluorescence microscope and cells were treated with different compounds through a perfusion system. A charge coupled device digital camera recorded signals emitted while switching exciting emission between 340nm and 380nm. Data were recorded as the ratio of 340nm:380nm by OptoFluor software to indicate fluctuations of $[Ca^{2+}]_i$. A physiological salt solution (Gey and Gey, 1936) (Table 2.6.1.2) containing 2mM glucose was used to establish a stable baseline of $[Ca^{2+}]_i$ and ATP, carbachol and/or tolbutamide were administered at the end of experiments as positive controls. 10µM O-1602, 5µM LPI and 1µM CBD were used as these concentrations have been previously shown to modify $[Ca^{2+}]_i$ in other cell types (Oka *et al.*, 2007; Waldeck-Weiermair *et al.*, 2008; Whyte *et al.*, 2009).

Reagent	Volume
BD Cell-Tak[®] Adhesive Reagent (in 5% acetic acid)	10 μ l
0.1M NaHCO₃ (pH 8)	285 μ l
1M NaCl	5 μ l

Table 2.6.1.1 Preparation of Cell-Tak[®]-coated cover-slips.

Cell-Tak[®] solution was made by the mixing reagents above in a 0.5ml Eppendorf tube and 1M NaCl was added immediately before the solution was deposited onto a 2.2cm circular acid/ethanol-washed sterile glass cover-slip. Cell-Tak[®] protein comes out of solution and spontaneously adsorbs to the cover-slips upon changing the pH from 2.4 to neutral. Cell-Tak[®] solution was kept on cover-slips at room temperature for 20 minutes to allow adsorption. Coated cover-slips were then washed twice with sterile DI H₂O, air dried and stored at 4°C prior to use.

Reagent	Molecular Weight	Amount for 2l	Final Concentration
NaCl	58.4g/mol	26g	111mM
KCl	74.6g/mol	1.48g	5mM
NaHCO₃	84.0g/mol	9.08g	27mM
MgCl₂.6H₂O	203.3g/mol	0.84g	1mM
KH₂PO₄	136.1g/mol	0.12g	0.22mM
MgSO₄.7H₂O	246.5g/mol	0.28g	0.28mM

Table 2.6.1.2 Preparation of 2x Gey and Gey buffer.

The Reagents listed above were dissolved in ~1.5l DI H₂O and adjusted to 2l with DI H₂O. The final pH should be ~8.4 before being diluted 1 in 2 in DI H₂O for use. To make 1x working buffer for calcium microfluorimetry experiments, HEPES and CaCl₂ solution were added giving final concentrations of 10.5mM and 2mM, respectively. The pH of the buffer was adjusted to 7.4 using 1M HCl.

2.6.2 RhoA activation assay

The RhoA Activation (Pull-down) Assay Kit™ from Cytoskeleton Inc. was used to measure activity of the small RhoA GTPase in MIN6 cells. The Rho binding domain (RBD) of Rho effector rhotekin protein binds specifically to GTP-bound RhoA and it is genetically-modified to be expressed as a GST-fusion protein bound to glutathione-sepharose beads. This allows ‘pull-down’ of activated RhoA and the amount of activated protein can be determined using SDS-PAGE and Western blotting analysis (Figure 2.6.2.1).

Serum-starved MIN6 cells were maintained as monolayers in 10cm Petri dishes between 50-70% confluency and were treated with a physiological salt solution (Table 2.6.1.1) containing 2mM glucose or 20mM glucose, in the presence or absence of agonist of interest for 10 minutes. Petri dishes were immediately placed on ice and protein extractions were carried out as described in Section 2.4.1. 10µl of extracted samples were removed for protein content quantification (Section 2.4.2) and the remaining samples were immediately snap-frozen in liquid nitrogen to avoid loss of RhoA activity. Samples were kept at -80°C and were defrosted in a 37°C water bath prior to use. 50µg of rhotekin-RBD beads was added to each sample and the mixtures were incubated at 4°C on a rotator for 1 hour. Beads were then pelleted by centrifugation at 10,000 rpm at 4°C for 3 minutes. Supernatants were carefully removed and the beads were washed once with wash buffer before adding the beads to 15µl NuPAGE® sample buffer. The samples were boiled for 2 minutes before being analysed by SDS-PAGE and Western blotting (primary antibody: anti-RhoA, 1:500 dilution, w/v; secondary antibody: anti-mouse, 1:5,000 dilution) (Section 2.4.3 and 2.4.4).

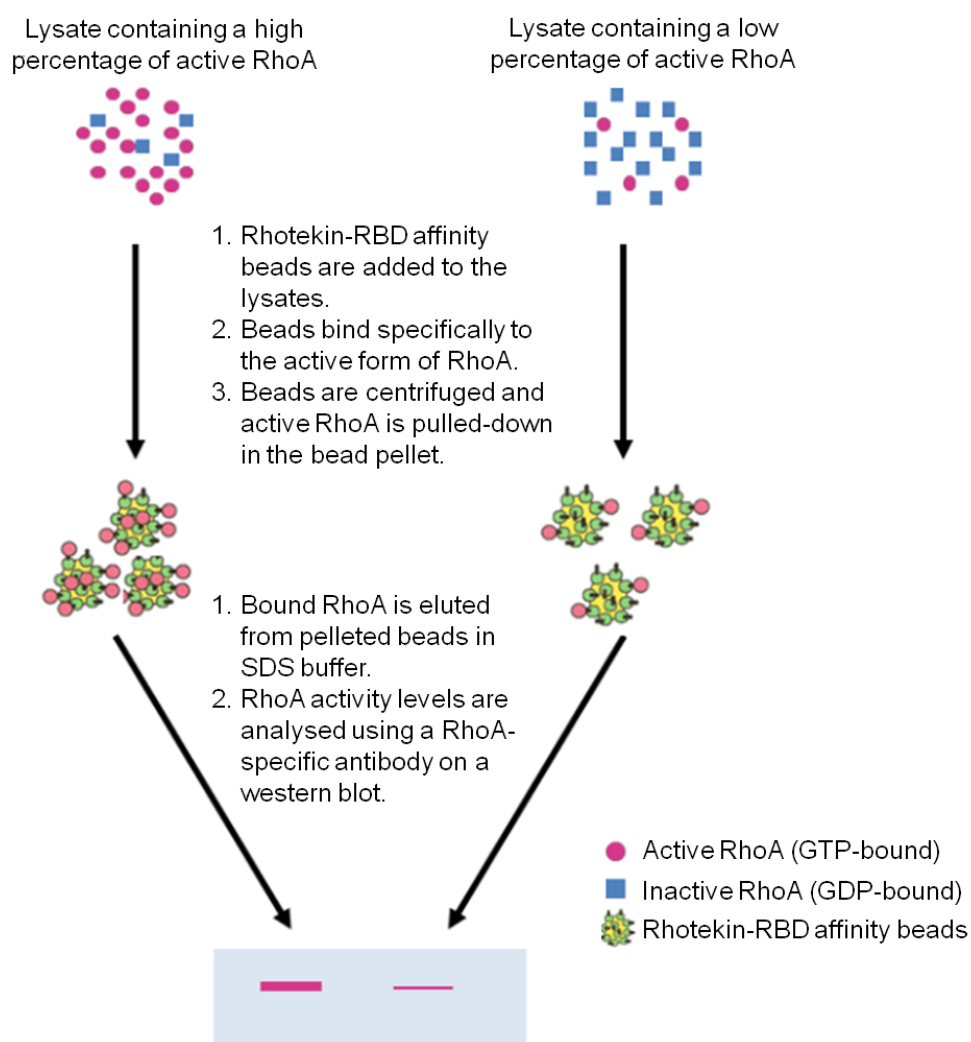


Figure 2.6.2.1 Schematic of RhoA Activation (Pull-down) Assay.

Rhotekin-RBD affinity beads were added to cell lysates and bound specifically to GTP-bound RhoA. Beads were then collected by centrifugation and bound RhoA was eluted from pelleted beads in SDS buffer. RhoA activity levels were then analysed by western blot using an anti-RhoA antibody. The amount of immunoreactive proteins reflected the amount of active RhoA in the original cell lysates. Diagram adapted from Cytoskeleton Inc. RhoA Activation Assay KitTM Manual (www.cytoskeleton.com/bk036).

2.7 Measurement of insulin secretion

2.7.1 Perifusion

Perifusion experiments were performed to measure dynamic insulin secretion from isolated mouse and human islets. The perifusion apparatus was set up in a temperature-controlled room at the optimal temperature for insulin secretion (37°C) and it consists of 16 chambers each containing a 1µm filter to retain the islets (Jones et al., 1986). Mouse or human islets were handpicked using a Gilson pipette into each chamber and pre-perifused for ~70 minutes with physiological salt buffer (Gey and Gey, 1936) containing 2mM glucose (Table 2.7.1.1). The pH of the buffer was adjusted to 7.4 by passing 95%CO₂/5%O₂ gas through the NaHCO₃-buffered solution. Islets were then perifused with physiological salt buffer supplemented with different concentrations of glucose and compounds of interest at a constant rate (0.5ml/min) using a peristaltic pump. Perifusates were collected every 2 minutes from series of tubes connected to the chambers and samples were assayed for insulin content by radioimmunoassay (Section 2.7.2).

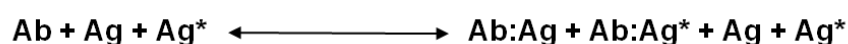
Reagent	Amount for 2l	Final Concentration
2x Gey and Gey buffer	1l	--
DI H ₂ O	1l	--
Glucose	0.72g	2mM
BSA	1g	0.5g/l
CaCl ₂ (1M)	4ml	2mM

Table 2.7.1.1 Preparation of Gey and Gey buffer for perifusion.

2x Gey and Gey buffer was prepared according to Table 2.6.1.1 and diluted 1 in 2 with DI H₂O. The prepared solution was first buffered to pH 7.4 using 95%CO₂/5%O₂ before adding the reagents listed above.

2.7.2 Insulin radioimmunoassay

Radioimmunoassay (RIA) uses a specific antibody to measure the amount of an antigen of interest *in vitro*. RIA was first applied to measure insulin concentration in 1960 (Yalow and Berson, 1960) and an assay based on the same principles were used in this thesis. The molecular basis of insulin RIA is competitive binding of radiolabelled insulin (Ag^*) or unlabelled insulin (Ag) to a rat anti-insulin antibody (Ab). A fixed, rate-limiting concentration of Ab was incubated with a mixture of ^{125}I -insulin (Ag^*) and defined concentrations of insulin (as standards) or unknown concentrations of insulin (as samples). The mixture was kept at 4°C for 48 hours to allow binding equilibrium between antibody and Ag^* or Ag as follows:



By keeping Ab and Ag^* constant, the concentration of Ab:Ag^* will vary according to the concentration of Ag . A standard curve can be plotted from which the insulin content of the samples can be determined.

In order to prepare a standard curve, standards of various mouse insulin concentrations (0.04, 0.08, 0.16, 0.32, 0.64, 1.25, 2.5, 5 and 10ng/ml) were prepared by serial dilution of 10ng/ml insulin stock in borate buffer (Table 2.7.2.1). 100 μl of insulin standards and samples were added to 200 μl of borate buffer containing ^{125}I -insulin tracer (total activity: 10,000 count per minute (cpm)) and anti-insulin antibody (1:10,000 dilution). When used at a final dilution of 1:10,000, the anti-insulin antibody had a detection limit of 8pg of insulin per tub, with intra- and inter-assay coefficients of variants of 5.2 and 14%, respectively as described before (Jones et al., 1988). Standards were assayed in triplicate and samples were assayed in duplicate. After 48 hours of incubation at 4°C , 1ml of precipitant (12% polyethylene glycol) was added before centrifugation (3,000rpm at 4°C , 15 minutes) to separate bound Ab:Ag^* complexes from free Ag^* molecules. Supernatant was aspirated off and the radioactivity of pellet was read using a Packard Wizard γ counter. A standard curve of radioactivities (cpm) from bound Ab:Ag^*

against insulin concentrations (ng/ml) was constructed and insulin contents of test samples were calculated from this curve by the γ counter software (Figure 2.7.2.1).

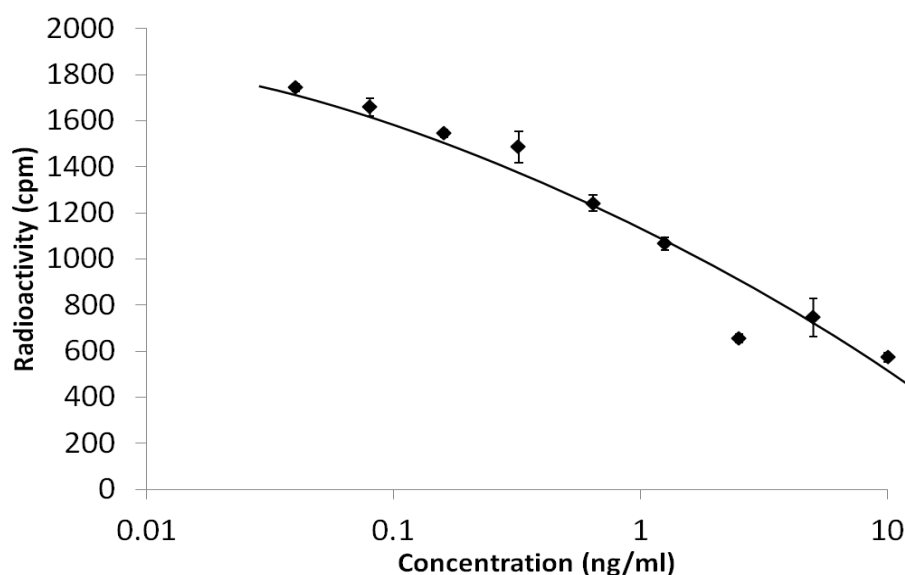


Figure 2.7.2.1 Typical standard curve for the insulin RIA.

Radioactivity (cpm) of each set of standards in triplicate was measured by a γ -counter and was plotted against corresponding insulin concentration to generate a standard curve. The insulin concentration axis was expressed as logarithm of 10 to allow linearisation of the standard curve. Insulin contents of test samples were calculated from this curve by the γ counter software.

Reagent	Molecular Weight	Amount for 2l	Final Concentration
Boric acid	61.8g/mol	16.5g	133mM
NaOH	40.0g/mol	5.4g	67.5mM
EDTA	292.2g/mol	7.4g	10mM

Figure 2.8.1.1 Preparation of Borate buffer.

The reagents listed above were dissolved in ~1.8l of DI H₂O. The pH was adjusted to 8 with concentrated HCl and the volume was made up to 2l with DI H₂O. 2g BSA was added before the solution was stored at 4°C.

2.8 Measurement of caspase activity

The Promega Caspase-Glo[®] 3/7 Assay is a luminescent assay that measures cell apoptosis *in vitro*. Cells in suspension were first transferred into wells of a 96-well cell culture plate followed by addition of the Caspase-Glo[®] 3/7 reagent. Cells were lysed immediately and proluminescent caspase-3/7 substrates in the reagent were cleaved enzymatically by active caspase-3 and -7 to release aminoluciferin. Aminoluciferin is a substrate of luciferase in the reagent to produce light. Amount of luminescent signals generated are proportional to the apoptosis level of lysed cells and it was recorded by a Veritas Microplate Luminometer.

Approximately 40-60 mouse islets were incubated in 2ml of RPMI medium supplemented with compounds of interest in a 35mm Petri dish for 48 hours under standard cell culture conditions. A cytokine cocktail (Table 2.8.1.1) was added to groups of islets 20 hours prior to the end of the incubation period to induce apoptosis. After 48 hours incubation, 8-11 sets of 3 islets were picked in 50µl RPMI medium and transferred into wells of a 96-well cell culture plate. Triplicates of 50µl of fresh RPMI medium were also transferred to 3 different wells for reference of background reading. 50µl of Promega Caspase-Glo[®] 3/7 reagent was added to each well and the plate was mixed for 1 minute on a plate shaker. The plate was then incubated at room temperature for 1 hour before acquisition of luminescent signals using the Veritas Microplate Luminometer. The value of mean background signal readings from RPMI medium was subtracted from each signal to give adjusted luminescent signals of each sample.

Cytokine	Stock Concentration	Amount	Final Concentration
IL-1β	100U/ μ l	1 μ l	0.05U/ μ l
TNF-α	1000U/ μ l	2 μ l	1U/ μ l
INF-γ	1000U/ μ l	2 μ l	1U/ μ l

Table 2.8.1.1 Preparation of cytokine cocktail.

5 μ l of cytokine cocktail was prepared by mixing all reagents listed above in a 0.5ml Eppendorf tube. The mixture was then transferred into the corresponding 35mm Petri dish containing islets and 2ml RPMI medium in the absence or presence of compounds of interest. Islets were incubated for approximately 20 hours to allow cytokine-induced apoptosis.

2.9 Statistical analysis

Numerical data are expressed as mean \pm standard error of mean (SEM) in this thesis. All statistical analyses was performed using Student's t-tests and P-values of <0.05 were considered statistically significant when comparing between different sets of data. For time course experiments where dynamic changes in intracellular calcium and insulin secretion were measured, data of the peak responses after addition of test compounds were statistically compared against the data at the time before the addition of the corresponding compounds.

Chapter 3 Expression of GPR55 by islets of Langerhans

CHAPTER 3 EXPRESSION OF GPR55 BY ISLETS OF LANGERHANS**3.1 Introduction**

The endocannabinoid system (ECS) consists of phospholipid-derived endocannabinoids, the G-protein coupled cannabinoid receptors and enzymes responsible for endocannabinoid biosynthesis and degradation. Three subtypes of cannabinoid receptors have been identified in mammals, including CB1 (Matsuda *et al.*, 1990), CB2 (Munro *et al.*, 1993) and the novel cannabinoid receptor GPR55 (Baker *et al.*, 2006). The CB1 receptor is widely expressed in the central nervous system (Pertwee *et al.*, 2010), as well as in some peripheral tissues including testes, prostate, heart, lung and bone marrow (Howlett *et al.*, 2002). The CB2 receptor is predominantly found in immune cells and it is expressed at a low level in other tissues (Munro *et al.*, 1993; Van Sickle *et al.*, 2005; Walter *et al.*, 2003). High levels of GPR55 mRNA expression have been detected in the central nervous system and in peripheral tissues, such as adrenals, jejunum, ileum and spleen (Ryberg *et al.*, 2007; Wu *et al.*, 2013). GPR55 mRNA has also been found in various cells types, including endothelial cells and Schwann cells (Daly *et al.*, 2010), osteoclasts and osteoblasts (Whyte *et al.*, 2009), and several cancer cell lines (Andradas *et al.*, 2011; Ford *et al.*, 2010; Pineiro *et al.*, 2011). GPR55 protein expression was detected by immunoblotting in human ovarian cancer cell line OVCAR3, human prostate cancer cell lines PC-3 and DU145 (Pineiro *et al.*, 2011).

The ECS has been shown to play an important role in regulating energy balance and metabolic homeostasis, and most studies have focusing on signalling via CB1 receptors in both central and peripheral tissues, including liver, adipose tissue, gastrointestinal tract, skeletal muscle and pancreas (Li *et al.*, 2011b). A number of studies have shown that the CB1 receptor is expressed by islets of Langerhans, but its cellular localisation within islets remains controversial (Bermudez-Silva *et al.*, 2008; Juan-Pico *et al.*, 2006; Li *et al.*, 2011a; Li *et al.*, 2010; Nakata and Yada, 2008; Tharp *et al.*, 2008). Less is known about the CB2 receptor due to its low expression level in non-immune cells, such as islet endocrine cells (Nakata and Yada, 2008;

Starowicz *et al.*, 2008; Tharp *et al.*, 2008). However, some studies have detected expression of the CB2 receptor in islets, but there is no consistency in reports on the localisation of this receptor (Bermudez-Silva *et al.*, 2008; Juan-Pico *et al.*, 2006; Li *et al.*, 2010; Starowicz *et al.*, 2008). The role of GPR55 in metabolic regulation has recently emerged: GPR55 mRNA expression has been detected in human visceral and subcutaneous adipose tissues, as well as in livers retrieved post mortem from both patients and normal controls (Moreno-Navarrete *et al.*, 2012). Another study has demonstrated that GPR55 mRNA expression was detectable in rat islets at a higher level than in rat liver and adipose tissue and its expression co-localised with islet β -cells, but not with α - or δ -cells (Romero-Zerbo *et al.*, 2011).

The aim of the experiments described in this chapter was to investigate the expression of GPR55 in MIN6 cells and in mouse and human islets of Langerhans.

3.2 Methods

3.2.1 RT-PCR

cDNAs from MIN6 cells and from mouse and human islets were obtained as described (Section 2.3.1). RT-PCR was performed using the primers designed for complementary binding with mouse and human GPR55 mRNAs. Detailed primer sequences, annealing temperatures and the number of amplification cycles are listed in Table 2.3.3.2. The PCR products were visualised under UV light after electrophoretic separation on 1.8% agarose gels containing 0.5 μ g/ml ethidium bromide.

3.2.2 SDS-PAGE and Western blotting

Protein samples of MIN6 cells, islets isolated from WT and GPR55 KO mice and human islets were prepared as described in Section 2.4.1 and protein content of each sample was quantified by the BCA assay (Section 2.4.2). Protein separation was carried out via SDS-PAGE and fractionated proteins were probed using a commercially available antibody raised in rabbit against the human GPR55 receptor. Immunoreactivities of GPR55 receptors were detected using enhanced chemiluminescence (ECL) after probing with an anti-rabbit secondary antibody (Section 2.4.3 and Section 2.4.4).

3.3 Results

3.3.1 Detection of mRNAs encoding GPR55 in MIN6 cells, mouse and human islets.

RT-PCR experiments identified expression of mRNA coding for the GPR55 receptor when the templates of cDNAs from MIN6 cells, mouse and human islets were used in amplifications (Figure 3.3.1.1). All PCR products were of the predicted size of 60bp and no product was amplified when cDNA was substituted with molecular biology grade water. Sequencing of the amplicons (by Source Bioscience) indicated 97-99% homology to the corresponding predicted nucleotide sequences.

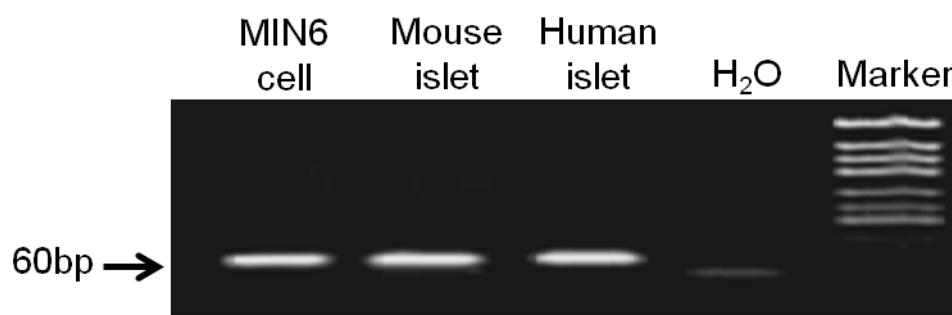


Figure 3.3.1.1 Detection of mRNAs encoding GPR55 in MIN6 cells, mouse and human islets.

Products of RT-PCR reactions were visualised shown under UV light after separation on a 1.8% agarose gel. Amplicons of the correct size (60bp) coding for GPR55 were amplified from MIN6 cells, mouse and human islet cDNAs. Molecular biology grade water was used as a negative control. The results shown here are representative of data collected from cDNAs prepared from 4 populations of MIN6 cells, 3 mice and 3 human islet donors.

3.3.2 Protein expression of GPR55 in MIN6 cells, mouse and human islets.

GPR55 is composed of 319 amino acids and has an expected molecular weight of 37kDa, and human GPR55 has 78% sequence homology with the murine protein (Ryberg *et al.*, 2007). Expression of GPR55 in islets was confirmed by immunoblotting analysis shown in Figure 3.3.2.1, which identified an immunoreactive protein of approximately 37kDa in protein extract from MIN6 cells, islets from WT mice and human islets, but not in protein extract from islets isolated from GPR55 KO mice. It can be seen that two additional immunoreactive proteins were also detected in the MIN6 cell and islet extract from WT mice, with molecular weights of 33kDa and 50kDa. The 33kDa protein might be a product of GPR55 degradation or it may represent a protein recognised by the GPR55 antibody through non-specific binding. Post-translational modifications, such as glycosylation can retard protein electrophoretic migration, and this may have resulted in the 50kDa immunoreactive protein that was identified. The 33kDa and 50kDa immunoreactive proteins were not detected in the islet extract obtained from GPR55 KO mice, confirming that they were either degradation or post-translational modification products of GPR55. In addition, an immunoreactive protein with molecular weight of 90kDa was identified in all samples and this is likely to have occurred through non-

specific binding of the primary antibody, since the same protein was also detected in islet extracts from GPR55 KO mice.

The commercially available anti-GPR55 antibody used here was raised against a human GPR55 protein fragment (antigen alignment sequence: ILLGRRDHTQDWV) and this sequence is not conserved in murine GPR55 (antigen alignment sequence: ILL_RRPDSTEDWV, underlined amino acids are not matched to the human GPR55). This may explain why a greater amount of immunoreactive protein was detected in human islets compared to the protein-matched MIN6 and mouse islet samples shown in Figure 3.3.2.1.

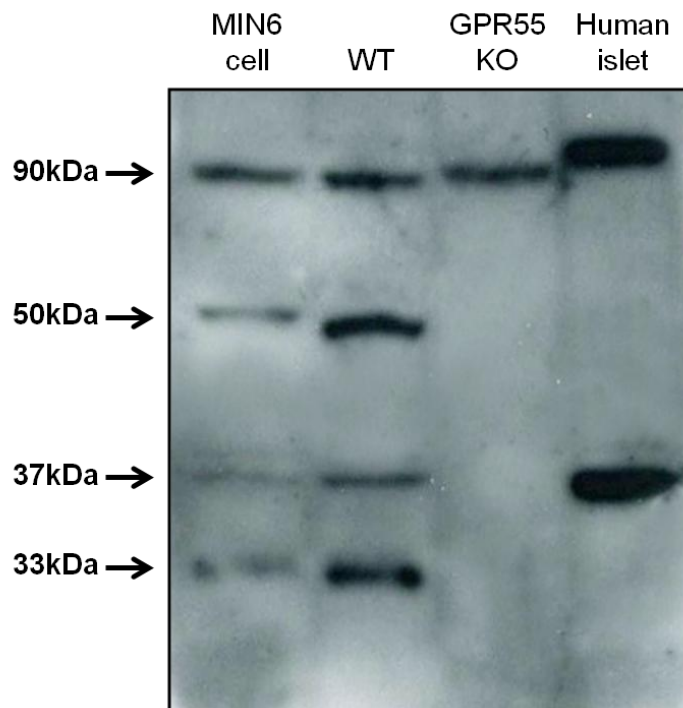


Figure 3.3.2.1 Protein expression of GPR55 in MIN6 cells, mouse and human islets.

An anti-human GPR55 antibody was used to identify GPR55 receptor expression in MIN6 cells, islets isolated from WT and GPR55 KO mice, and human islets. 20µg of each sample were loaded onto 10% polyacrylamide gels and immunoreactive proteins were detected by ECL. The blots shown here are representative of 4 experiments using samples obtained from 5 populations of MIN6 cells, 3 WT and GPR55 KO mice and 2 human islet donors.

3.4 Discussion

GPR55 has been suggested to play important roles in the cardiovascular system, bone metabolism, inflammation, neuronal function and cancer proliferation. (Andradas *et al.*, 2011; Daly *et al.*, 2010; Obara *et al.*, 2011; Pineiro *et al.*, 2011; Staton *et al.*, 2008; Whyte *et al.*, 2009). Very little is known about the role of GPR55 in islets, apart from two papers suggested that GPR55 regulates insulin secretion from islets (McKillop *et al.*, 2013; Romero-Zerbo *et al.*, 2011). However, more research is needed to validate the finding in rodents and to explore the importance of GPR55 in the regulation of human metabolism. The aim of this thesis is to identify the roles of GPR55 in islet functions. So it was therefore important to investigate if the receptor is expressed by MIN6 cells, mouse and human islets which are the models that will be used in subsequent functional studies in the following chapters.

The data in this chapter, showing that GPR55 is expressed at both mRNA and protein levels in MIN6 cells, mouse and human islets, are consistent with the previous study and demonstrate for the first time, that GPR55 is expression by human islets. The immunoblotting analysis, where products larger and smaller than the 37kDa predicted sequence of GPR55 were detected, indicate the possible presence of GPR55 degradation and post-translational modification products in mouse islets. It will be interesting to perform parallel experiments with protein extracts from other mouse tissues to investigate whether these findings are islet-specific. An earlier study has demonstrated that GPR55 mRNA is expressed by rat islets and immunostaining analysis has detected GPR55 in β -cells from paraffin-fixed rat pancreas (Romero-Zerbo *et al.*, 2011). This is in agreement with another immunostaining experiment showing that mouse β -cells expressed GPR55, while there was no evidence of the receptor in α -cells (McKillop *et al.*, 2013).

Generation of second messengers, such as intracellular calcium, are common downstream signalling mechanisms of GPCRs in regulating islet functions and it has been shown that

GPR55 couples to Gq α protein to elevate intracellular calcium concentration (Ross, 2009; Sharir and Abood, 2010). In addition, GPR55 has been shown to activate another class of second messengers, Rho small GTPases, which are involved in reorganisation and remodelling of filamentous actin in the regulation of insulin release (Kalwat et al., 2013; Ross, 2009; Sharir and Abood, 2010). The following chapter presents data showing how the second messenger levels in islet cells change in response to pharmacological manipulations of GPR55.

Chapter 4 Pharmacological manipulation of GPR55 in islets: Second messenger generation

CHAPTER 4 PHARMACOLOGICAL MANIPULATION OF GPR55 IN ISLETS:**SECOND MESSENGER GENERATION****4.1 Introduction**

Expression studies in the last chapter have shown that GPR55 is expressed by islets of Langerhans. In order to investigate if GPR55 plays a role in regulating islet functions, it is important to understand the signalling cascade downstream of GPR55 in islets. Studies in other cell types have suggested that GPR55 is coupled to either Gq or G12/13 α subunits (Ross, 2009; Sharir and Abood, 2010). Activation of the Gq α subunit is linked to elevations in intracellular calcium ($[Ca^{2+}]_i$) through the PLC/PKC signalling pathway and it has been well established that activation of GPR55 stimulates generation of this second messenger (Ross, 2009; Sharir and Abood, 2010). In addition, experiments using the GPR55-transfected human embryonic kidney cell line HEK293 have shown that pharmacological activation of GPR55 by cannabinoids and other phospholipid-derived compounds induced elevations in $[Ca^{2+}]_i$ and pharmacological inhibition and siRNA-knockdown of GPR55 expression abolished these effects (Henstridge *et al.*, 2010; Oka *et al.*, 2007; Waldeck-Weiermair *et al.*, 2008). Increases in $[Ca^{2+}]_i$ after administration of GPR55 agonists were also reported in primary endothelial cells (Kargl *et al.*, 2013), cultured neonatal cardiomyocytes (Yu *et al.*, 2013), the PC-3 human prostate cancer cell line (Pineiro *et al.*, 2011) and primary CA3 pyramidal cells (Sylantsev *et al.*, 2013).

The GPR55 agonist O-1602 is widely used as a specific activator of the receptor. It was able to elicit increases in $[Ca^{2+}]_i$ (Sylantsev *et al.*, 2013; Waldeck-Weiermair *et al.*, 2008) and knockdown of GPR55 with siRNA diminished O-1602-induced Ca^{2+} signalling in endothelial cells (Waldeck-Weiermair *et al.*, 2008). Moreover, lysophospholipid, lysophosphatidylinositol (LPI), was reported to unexpectedly activate GPR55 in 2007 (Oka *et al.*, 2007) and

accumulating research has suggested that LPI is an endogenous GPR55 ligand (Ross, 2009; Sharir and Abood, 2010). However, it remains controversial that the effects of LPI on $[Ca^{2+}]_i$ were mediated through GPR55. Thus, while some studies demonstrated that GPR55 inhibitors and knockdown of GPR55 gene abolished the effect of LPI on $[Ca^{2+}]_i$ (Oka *et al.*, 2007; Pineiro *et al.*, 2011; Sylantsev *et al.*, 2013; Waldeck-Weiermair *et al.*, 2008), other studies reported GPR55-independent effects of LPI on $[Ca^{2+}]_i$ (Bondarenko *et al.*, 2010). Subsequent studies suggested that LPI regulated $[Ca^{2+}]_i$ via acting on Ca^{2+} -activated K^+ channels (Bondarenko *et al.*, 2011a, b).

In addition to activating the Gq α subunit, GPR55 was also suggested to be linked to the G12/13 α subunit by the demonstration that an anti- G12/13 peptide was able to reduce O-1602-induced GPR55 signalling in a concentration-dependent manner (Ryberg *et al.*, 2007). The G12/13 α subunit is known to activate a second messenger family of Rho small GTPases, including RhoA, Cdc42 and Rac1 (Fromm *et al.*, 1997). It has been reported that O-1602-induced activation of GPR55 caused RhoA activation in HEK293 cells and osteoclasts (Ryberg *et al.*, 2007; Whyte *et al.*, 2009), and these effects were inhibited by antagonising GPR55 and *in vivo* knockdown of GPR55 (Ryberg *et al.*, 2007; Whyte *et al.*, 2009). LPI-induced activations of RhoA were also demonstrated separately in osteoclasts and also in the PC12 rat adrenal medulla cell line (Obara *et al.*, 2011; Whyte *et al.*, 2009).

Increases in $[Ca^{2+}]_i$ are involved in both first and second phases of glucose-induced insulin secretion (Jing *et al.*, 2005; Rorsman and Renstrom, 2003; Schulla *et al.*, 2003). Some receptor ligands such as acetylcholine can also potentiate glucose-induced insulin secretion from β -cells via activating Gq-coupled GPCRs and elevating $[Ca^{2+}]_i$. In addition, increased calcium influx is linked to the regulation of transcription factors important to islet survival, such as CREB and NFAT (Liu *et al.*, 2012; Soleimanpour *et al.*, 2010). Interestingly, some studies suggest that RhoA and its downstream effector Rho-associated kinase (ROCK) have a negative

consequence on insulin secretion, but their roles on other islet functions remain unknown (Hammar *et al.*, 2009; Tomas *et al.*, 2010). In order to find out the roles of GPR55 in islets, it is important to understand how this receptor regulates these two second messenger pathways in islets.

Experiments described in this chapter therefore investigated the effects of O-1602, LPI and the GPR55 antagonist cannabidiol (CBD) on regulating $[Ca^{2+}]_i$ and RhoA in MIN6 β -cells and primary mouse and human islets. The specificity of these agents on modulating $[Ca^{2+}]_i$ was also explored using islets isolated from GPR55 KO mice.

4.2 Methods

4.2.1 Calcium microfluorimetry

Approximately 30,000 MIN6 β -cells (p31-40) were seeded onto 2.2cm circular acid/ethanol-washed Cell-Tak[®]-coated sterile glass coverslips (Section 2.6.1). Around 100 islets isolated from WT and GPR55 KO mice, or 100 isolated human islets, were dispersed using Cell Dissociation Solution before seeding in the same procedure for MIN6 cells. After 24 hours in culture, Fura-2 AM was added to the culture medium (final concentration: 5 μ M), and cells were incubated for 30 minutes prior to experiments. The Fura-2-loaded cells were then perfused with physiological buffer (Gey and Gey, 1936) in the presence of compounds of interest and appropriate positive controls. Cells were excited with 340nm and 380nm wavelengths and the fluorescence intensity, recorded at 510nm as a ratio of 340nm/380nm, was used as a measurement of $[Ca^{2+}]_i$.

4.2.2 RhoA activation assay

MIN6 cells grown to 50~70% confluence in 90mm Petri dishes were serum-starved overnight in DMEM supplemented with 5mM glucose and penicillin / streptomycin. On the next day, DMEM was replaced with 10ml physiological buffer (Gey and Gey, 1936) containing 2mM or

20mM of glucose in the absence or presence of 10 μ M O-1602 and cells were incubated at 37°C for 10 minutes. Petri dishes were immediately placed on ice and protein extractions were carried out as described in Section 2.4.1. 10 μ l of extracted samples were removed for protein content quantification (Section 2.4.2) and total RhoA activity measurements and the remaining samples were immediately snap-frozen in liquid nitrogen to avoid loss of RhoA activity. Samples were thawed in a water bath at 37°C, 50 μ g rhotekin-RBD beads was added to each sample and the mixtures were incubated at 4°C on a rotator for 1 hour. Beads were then pelleted by centrifugation and washed once with wash buffer before adding 15 μ l NuPAGE® sample buffer. The samples were then analysed by SDS-PAGE and Western blotting (primary antibody: anti-RhoA, 1:500 dilution; secondary antibody: anti-mouse, 1:5,000 dilution, Section 2.4.3 and 2.4.4).

4.3 Results

4.3.1 Effects of pharmacological manipulation of GPR55 on [Ca²⁺]_i in MIN6 cells.

The effects of pharmacological manipulation of GPR55 on [Ca²⁺]_i were first studied in MIN6 cells. 10 μ M O-1602 and 5 μ M LPI were used as these concentrations have been previously shown to elevate [Ca²⁺]_i (Oka *et al.*, 2007; Waldeck-Weiermair *et al.*, 2008). 1 μ M CBD since this concentration has previously been shown to inhibit O-1602- and LPI-mediated elevations in [Ca²⁺]_i (Whyte *et al.*, 2009). MIN6 cells were also challenged with two positive control agonists at the end of each experiment to demonstrate that they were still viable. The purinergic receptor agonist ATP (100 μ M) was used as a positive control for GPCR-mediated increases in [Ca²⁺]_i via PLC activation in β -cells (Hauge-Evans *et al.*, 2002; Squires *et al.*, 1995). In addition, tolbutamide (100 μ M) was used as another positive control since it is able to depolarise β -cell through inhibition of K_{ATP}-channel via interaction with the SUR1 subunit (Hatlapatka *et al.*, 2009).

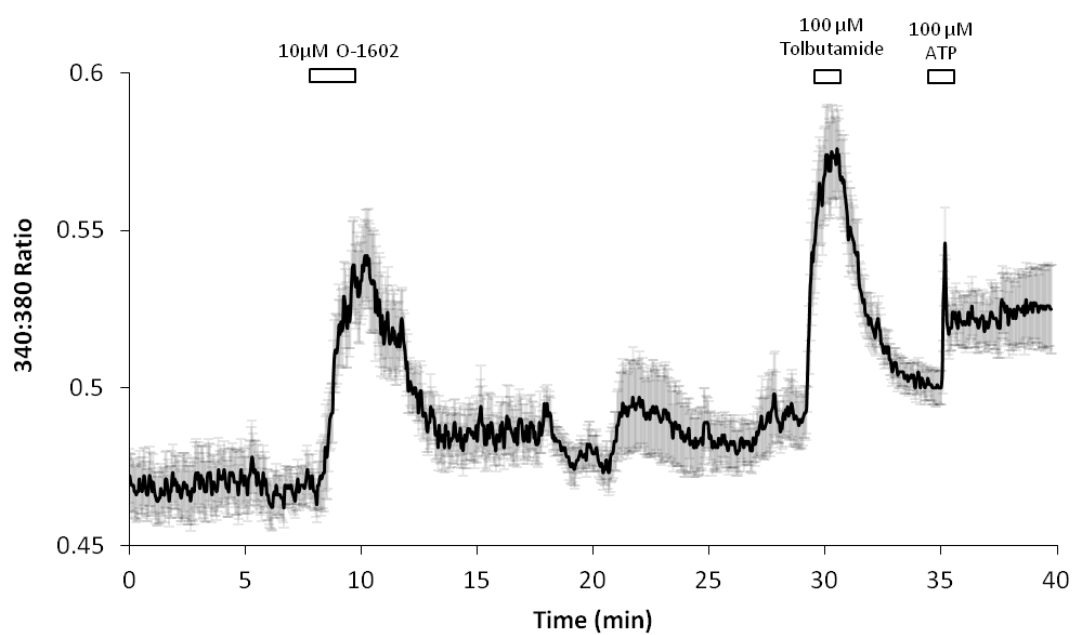
Administration of ATP and tolbutamide caused immediate and reversible increases in [Ca²⁺]_i in Fura-2-loaded MIN6 cells. The effect of ATP was transient since [Ca²⁺]_i decreased toward

basal level before the withdrawal of ATP, while the effect of tolbutamide on $[Ca^{2+}]_i$ was long lived. It is worth noting from Figure 4.3.1.1B and 4.3.1.1C that ATP- and tolbutamide-induced elevation in $[Ca^{2+}]_i$ peaked at a similar amplitude when cells were exposed to ATP before tolbutamide. However, if the cells were treated with ATP after tolbutamide, only a small and transient $[Ca^{2+}]_i$ increase was observed as illustrated in Figure 4.3.1.1A ($64 \pm 19\%$, peak stimulation as a percentage of maximum response to $100\mu\text{M}$ tolbutamide, mean \pm SEM, $n=10$). The reason for the reduced response to ATP may be that a limited amount of intracellular Ca^{2+} was available after tolbutamide stimulation, via calcium-induced calcium release from the endoplasmic reticulum.

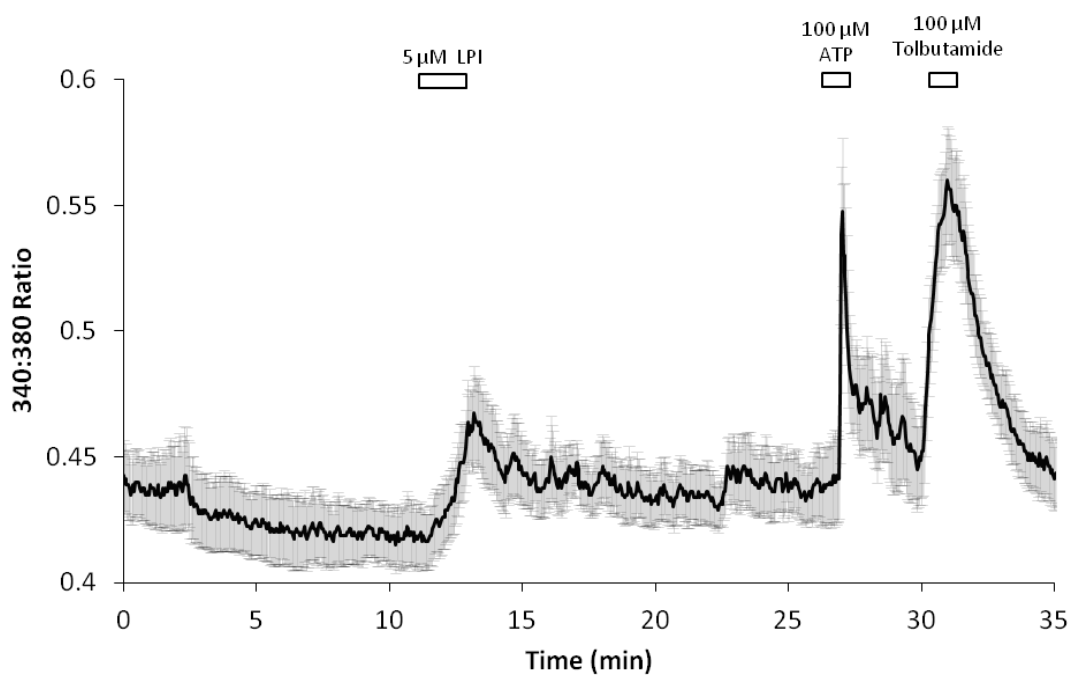
Figure 4.3.1.1A demonstrates that $10\mu\text{M}$ O-1602 was able to induce a robust increase in $[Ca^{2+}]_i$ in MIN6 cells ($106 \pm 28\%$, of maximum response to $100\mu\text{M}$ tolbutamide, mean \pm SEM, $n=10$). The response was maintained in the presence of O-1602 and returned towards basal after its removal. Further experiments, as shown in Figure 4.3.1.1B, demonstrated that $5\mu\text{M}$ LPI also caused a reversible and long-lived elevation in $[Ca^{2+}]_i$ in MIN6 cells. However, amplitude of the response to LPI was considerably less than that of O-1602. ($50 \pm 12\%$, of maximum response to $100\mu\text{M}$ tolbutamide, mean \pm SEM, $n=8$).

CBD has been reported to cause a small, but insignificant increase in $[Ca^{2+}]_i$ in HEK293 cells overexpressing GPR55 (Lauckner *et al.*, 2008), but it was suggested to be an antagonist of GPR55 in various studies where it inhibited the physiological effects of O-1602 and LPI (Li *et al.*, 2013; Ryberg *et al.*, 2007; Whyte *et al.*, 2009). Before testing how CBD would affect O-1602- and LPI-induced changes in $[Ca^{2+}]_i$, the effect of CBD alone on $[Ca^{2+}]_i$ of MIN6 cells was studied first. Unexpectedly, addition of $1\mu\text{M}$ CBD resulted in elevation in $[Ca^{2+}]_i$ in MIN6 cells, which was slow in onset and slowly reversible after removal of CBD (Figure 4.3.1.1C, $88 \pm 48\%$ of maximum response to $100\mu\text{M}$ tolbutamide, mean \pm SEM, $n=17$). The stimulatory

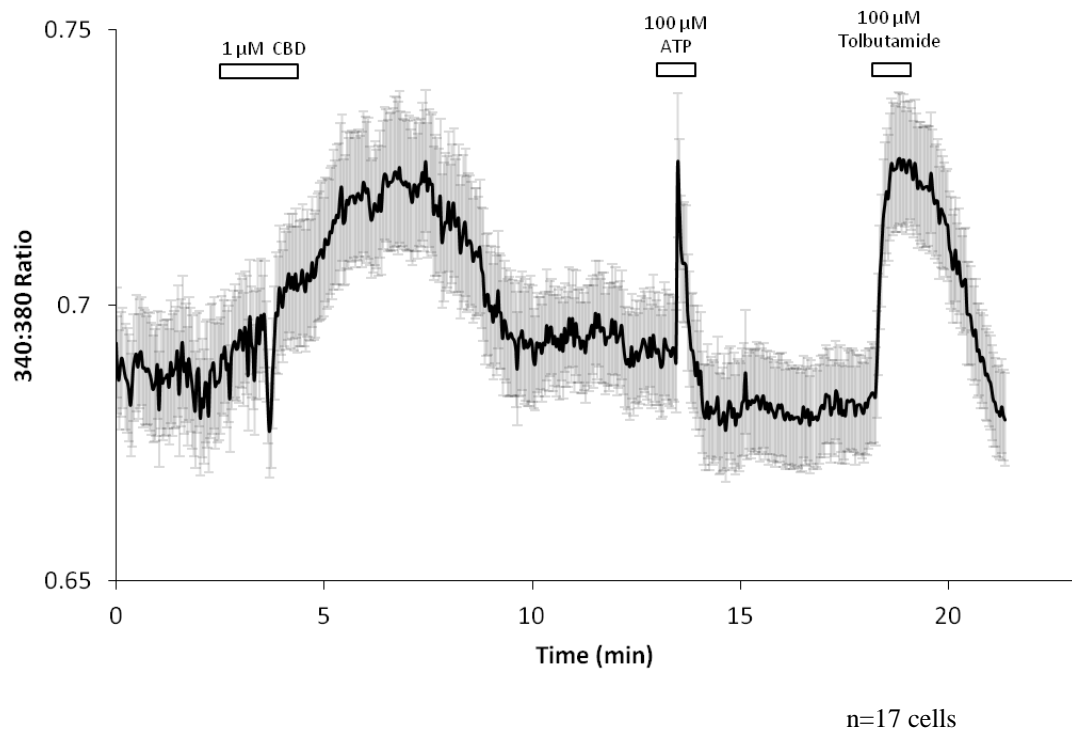
effect of CBD means that it cannot be used as an antagonist of GPR55 for measurement of Ca^{2+} in islet cells.

A MIN6 cells with O-1602

n=10 cells

B MIN6 cells with LPI

n=8 cells

C MIN6 cells with CBD**Figure 4.3.1.1 Effects of O-1602, LPI and CBD on $[Ca^{2+}]_i$ in MIN6 cells.**

Fura-2-loaded MIN6 cells were perfused with physiological buffer containing 2mM glucose and supplemented with O-1602 (A), LPI (B), CBD (C) and appropriate positive controls (100μM ATP and 100μM tolbutamide), as shown. Changes in $[Ca^{2+}]_i$ were measured by single-cell calcium microfluorimetry and expressed as a ratio of 340nm/380nm fluorescence. Results shown here are mean values of individual cells within a single field of view representative of at least 5 different experiments. Data are presented as mean \pm SEM.

4.3.2 Effect of pharmacological manipulation of GPR55 on $[Ca^{2+}]_i$ in dispersed mouse islet cells from WT and GPR55 KO mice.

The preceding data demonstrated that O-1602 increased $[Ca^{2+}]_i$ in MIN6 cells so further experiments were performed to determine whether the agonist would be able to induce the same effect in mouse islet primary cells. Data presented here represent $[Ca^{2+}]_i$ measurements from single cells due to the heterogeneity of responses recorded from dispersed mouse islet cells. Only approximately 50% of seeded cells responded to 20mM glucose, 500 μ M carbachol and 100 μ M tolbutamide and they were considered to be islet β -cells, since they showed the characteristic changes in $[Ca^{2+}]_i$ in response to these agonists. The results presented in Figure 4.3.2.1A demonstrate that 10 μ M O-1602 was able to induced an increase in $[Ca^{2+}]_i$ in dispersed mouse islet cells at 2mM glucose as it did in MIN6 cells. However, the effect was observed only in 50% of glucose/carbachol/tolbutamide-positive islet cells. In addition, Figure 4.3.2.1C demonstrated that O-1602 was also able to potentiate glucose-induced increase in $[Ca^{2+}]_i$ and 33% of β -cells responded to the agonist at 20mM glucose.

In order to investigate if O-1602 acted specifically on GPR55 to mediate its effects on β -cell $[Ca^{2+}]_i$, further experiments were conducted using dispersed islet cells from GPR55 KO mice. Representative Ca^{2+} traces shown in Figure 4.3.2.1B and D show that O-1602 did not stimulate elevation in $[Ca^{2+}]_i$ in the absence of GPR55 and analysis of all data indicated that only 7% of β -cells responded to O-1602 at 2mM glucose after GPR55 deletion, and none responded at 20mM glucose.

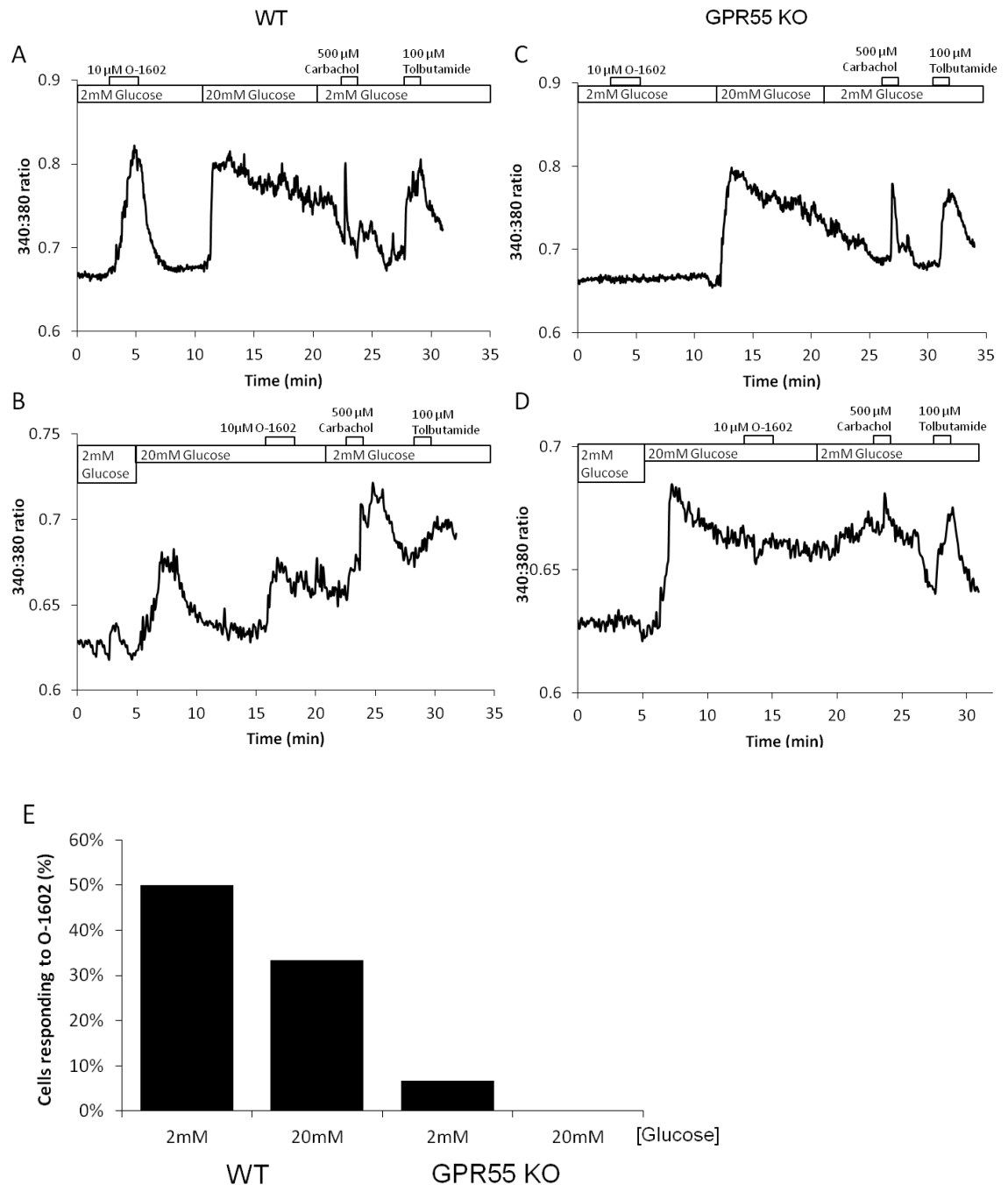


Figure 4.3.2.1 O-1602 increases $[Ca^{2+}]_i$ in islet β -cells from WT mice, but not in cells from GPR55 KO mice.

Fura-2-loaded mouse islet cells were perfused with physiological buffer containing 2mM glucose and supplemented as shown. Addition of O-1602 (10 μ M, 4-6 min) at 2mM glucose caused an elevation in $[Ca^{2+}]_i$ in β -cells from WT mice (A), but not in β -cells from GPR55 KO mice (C). Similar effects on $[Ca^{2+}]_i$ by O-1602 (10 μ M, 15-17 min) at 20mM glucose were also observed from β -cells from WT mice (B), but not in β -cells from GPR55 KO mice (D). Islet β -cells were characterised by responding to 20mM glucose, 0.5mM carbachol and 100 μ M tolbutamide. Results shown here are single cell calcium traces representing at least 10 cells from one experiment. E Percentage of β -cells from WT and GPR55 KO mice that responded to O-1602 at either 2mM or 20mM glucose. Data are representative of 4 separate experiments.

Further experiments were performed to investigate the effect of LPI on $[Ca^{2+}]_i$ in β -cells from WT and GPR55 KO mice. Similar to O-1602, Figure 4.3.2.2A and B illustrated that 5 μ M LPI induced increases in $[Ca^{2+}]_i$ at both basal and stimulatory glucose levels in β -cells from WT mice. The results here agreed with observations of the effects of LPI on $[Ca^{2+}]_i$ in MIN6 cells at 2mM glucose from Figure 4.3.1.1B, but the magnitude of its effect was greater in dispersed mouse β -cells. The same proportion of β -cells (65%) responded to LPI at 2mM and 20mM glucose. In contrast to the lack of effects of O-1602 observed in β -cells from GPR55 KO mice, Figure 4.3.2.2C and 4.3.2.2D demonstrated that LPI induced elevations in $[Ca^{2+}]_i$ at both 2mM and 20mM glucose in β -cells from the same group of mice. Figure 4.3.2.2E illustrated that, similar to the proportion of β -cells from WT mice that responded to LPI, 62% and 67% of β -cells from GPR55 KO mice had elevations in $[Ca^{2+}]_i$ after administration of LPI at 2mM and 20mM glucose, respectively.

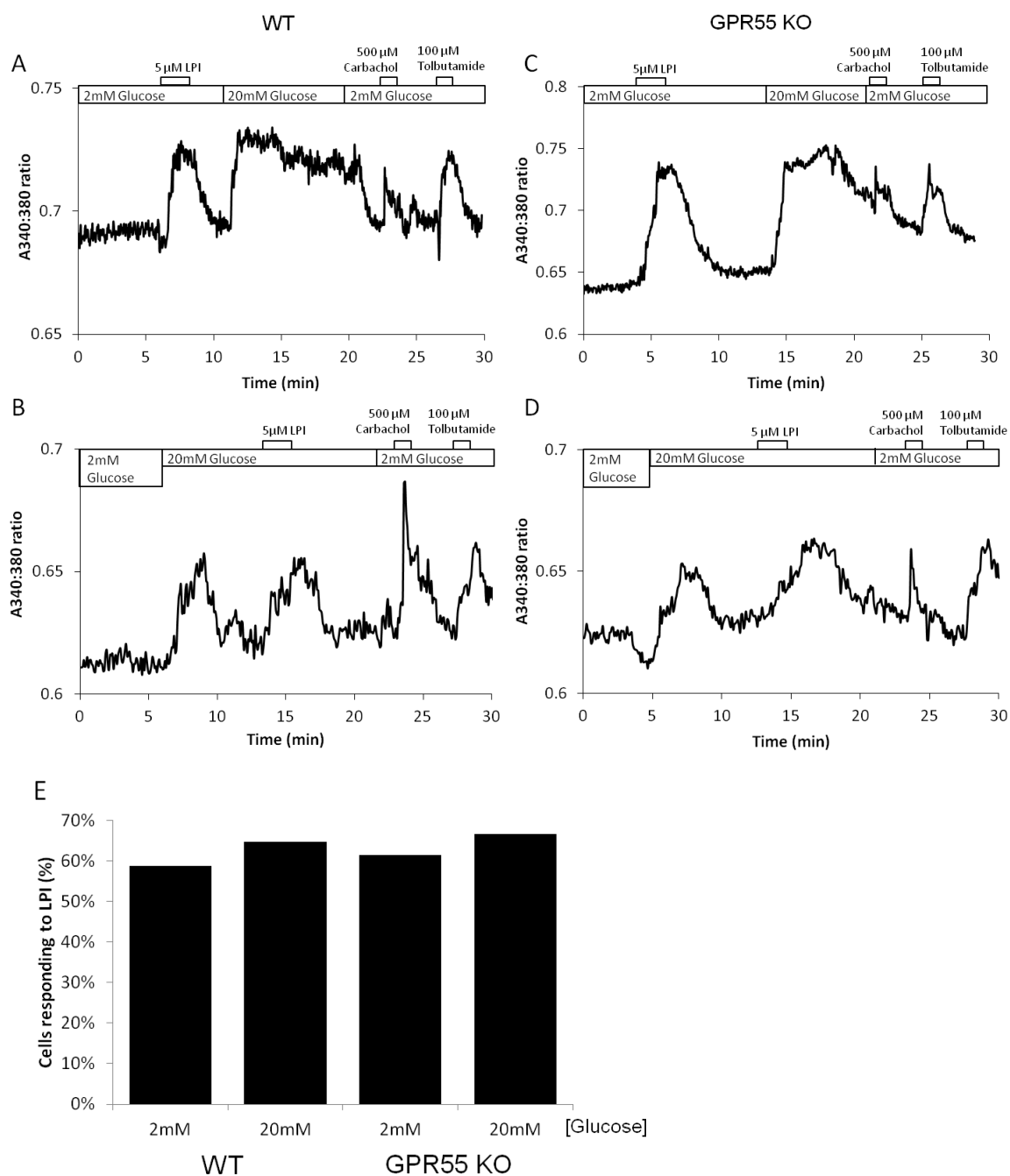


Figure 4.3.2.2 LPI increases $[Ca^{2+}]_i$ in islet β -cells from both WT and GPR55 KO mice.

Fura-2-loaded mouse islet cells were perfused with physiological buffer containing 2mM glucose and supplemented as shown. Addition of LPI (5 μ M, WT: 6-8 min; GPR55 KO: 5-7 min) at 2mM glucose caused an elevation in $[Ca^{2+}]_i$ in β -cells from both WT (A) and GPR55 KO mice (C). Similar effects on $[Ca^{2+}]_i$ by LPI (5 μ M, 14-16 min) at 20mM glucose were also observed in β -cells from WT (B) and GPR55 KO mice (D). Islet β -cells were characterised by responding to 20mM glucose, 0.5mM carbachol and 100 μ M tolbutamide. Results shown here are single cell calcium traces representing at least 10 cells from one experiment. E Percentage of β -cells from WT and GPR55 KO mice that responded to LPI at either 2mM or 20mM glucose. Data are representative of 4 separate experiments.

My experiments in MIN6 cells unexpectedly demonstrated that CBD was able to induce elevations in $[Ca^{2+}]_i$, inconsistent with its antagonising effects on GPR55 reported in other studies. To determine whether that stimulatory effects occurred via activation of GPR55, further experiments were performed using islets isolated from WT and GPR55 KO mice. 55% and 50% of β -cells from WT mice responded to CBD with increases in $[Ca^{2+}]_i$ at 2mM and 20mM glucose, respectively (Figure 4.3.2.3 A and B). The magnitude of response observed at 2mM glucose in β -cells is similar to that observed in MIN6 cells as shown in Figure 4.3.1.1C. These effects were still observed in β -cells from GPR55 KO mice, as illustrated in Figure 4.3.2.3C and D. It is interesting to note that more β -cells from GPR55 KO mice (56% at 2mM glucose and 67% at 20mM glucose) responded to CBD with an elevation in $[Ca^{2+}]_i$ (Figure 4.3.2.3E), possibly due to an upregulation of gene expression of the true target protein(s) of CBD after GPR55 deletion.

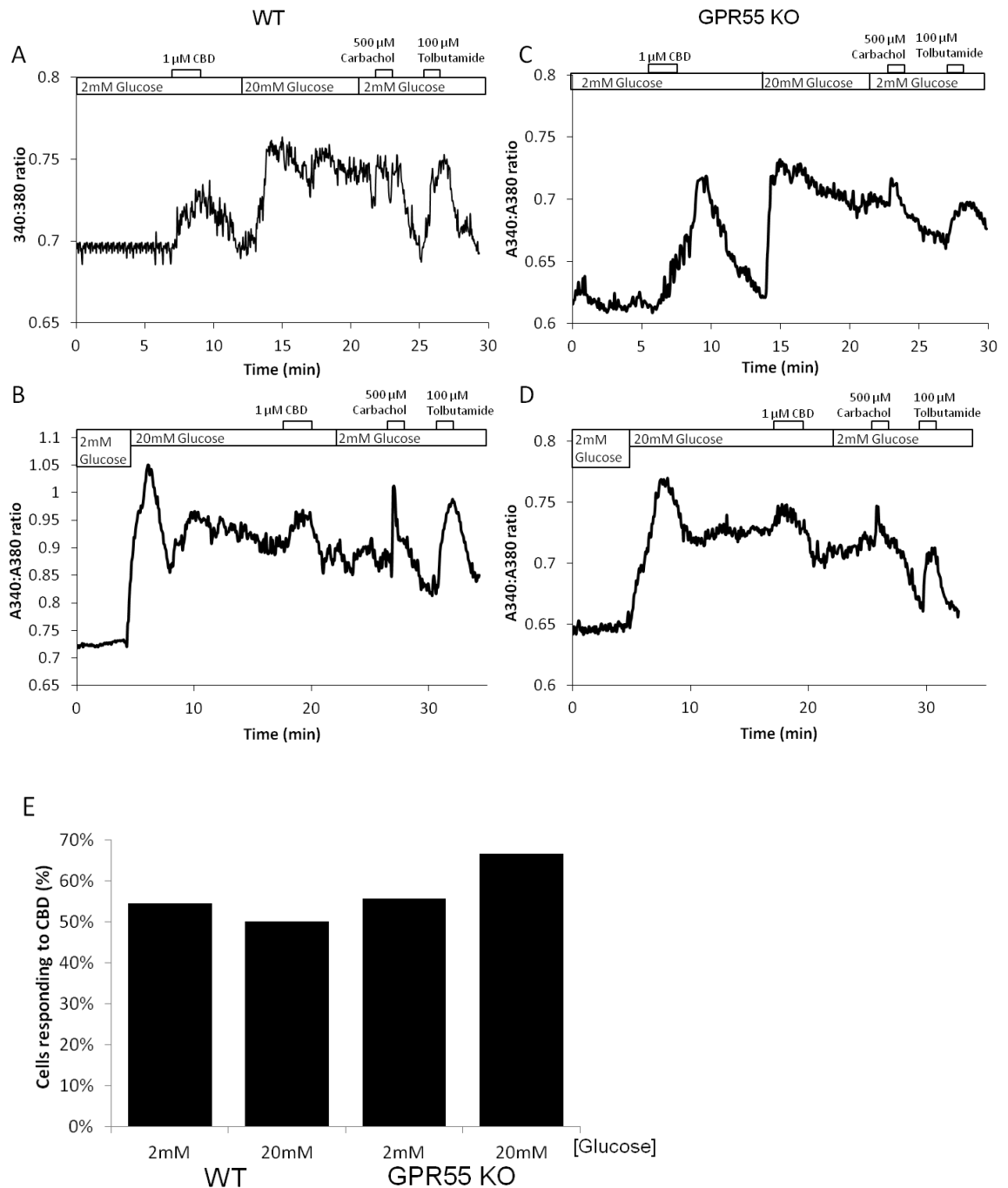


Figure 4.3.2.3 CBD increases $[Ca^{2+}]_i$ in islet β -cells from both WT and GPR55 KO mice. Fura-2-loaded mouse islet cells were perfused with physiological buffer containing 2mM glucose and supplemented as shown. Addition of CBD (1 μ M, 6-8 min) at 2mM glucose caused an elevation in $[Ca^{2+}]_i$ in β -cells from both WT (A) and GPR55 KO mice (C). Similar effects on $[Ca^{2+}]_i$ by CBD (1 μ M, 18-20 min) at 20mM glucose were also observed in β -cells from WT (B) and GPR55 KO mice (D). Islet β -cells were characterised by responding to 20mM glucose, 0.5mM carbachol and 100 μ M tolbutamide. Results shown here are single cell calcium traces representing at least 10 cells from one experiment. E Percentage of β -cells from WT and GPR55 KO mice that responded to CBD at either 2mM or 20mM glucose. Data are representative of 4 separate experiments.

4.3.3 Effect of activation of GPR55 on $[Ca^{2+}]_i$ in dispersed human islets cells.

Since O-1602 and LPI stimulated increases in $[Ca^{2+}]_i$ in MIN6 cells and mouse β -cells, it was important to determine if these ligands also trigger the same responses in human islet cells. In order to further investigate the effect of O-1602 and LPI on regulating calcium handling in human islet cells, isolated human islets were dispersed as described in Section 2.6.1 and loaded with Fura-2 for calcium microfluorimetry. In accordance with the data generated from MIN6 cells and mouse β -cells, Figure 4.3.3.1 shows that 10 μ M O-1602 and 5 μ M LPI were able to induce elevations in $[Ca^{2+}]_i$. similar to dispersed mouse islet cells, data presented here were also $[Ca^{2+}]_i$ measurement from single cell due to the heterogeneity of responses recorded from dispersed human islet cells. The response of O-1602 in dispersed human islets cells was early onset and dismissed after O-1602 removal, but the magnitude of the effect was considerable smaller compared to that in MIN6 cells (Figure 4.3.1.1A) and mouse β -cells (Figure 4.3.2.1A). On the other hand, the response of LPI was also early onset, but maintained after the withdrawal of LPI. The magnitude of the effect of LPI at 2mM glucose was similar to 100 μ M tolbutamide in dispersed human islet cells, which was not observed in MIN6 cells (Figure 4.3.1.1B) and mouse β -cells (Figure 4.3.2.2A). The effect of CBD on $[Ca^{2+}]_i$ in dispersed human islets cells was not investigated due to the time constraint of the project.

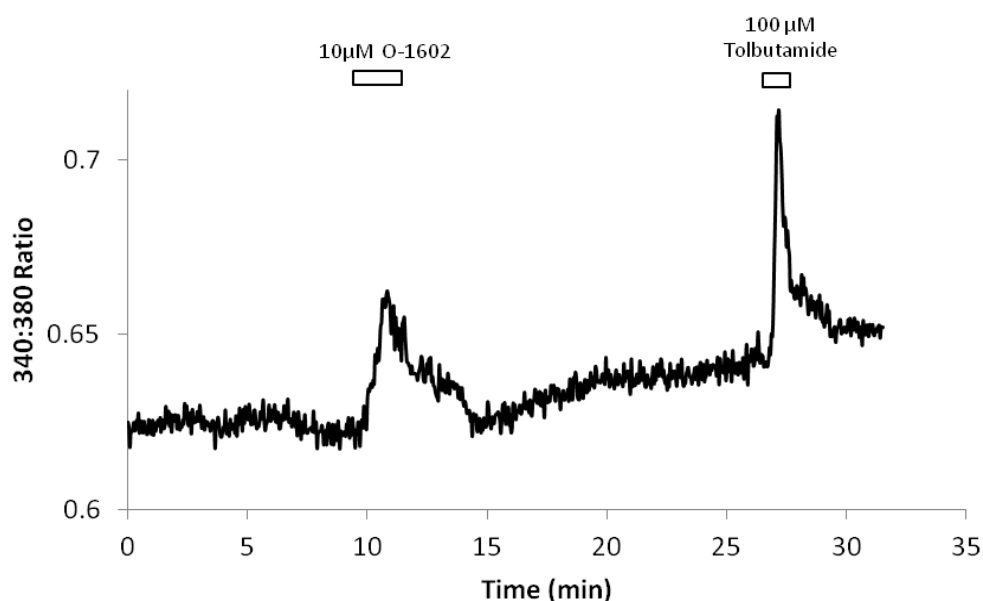
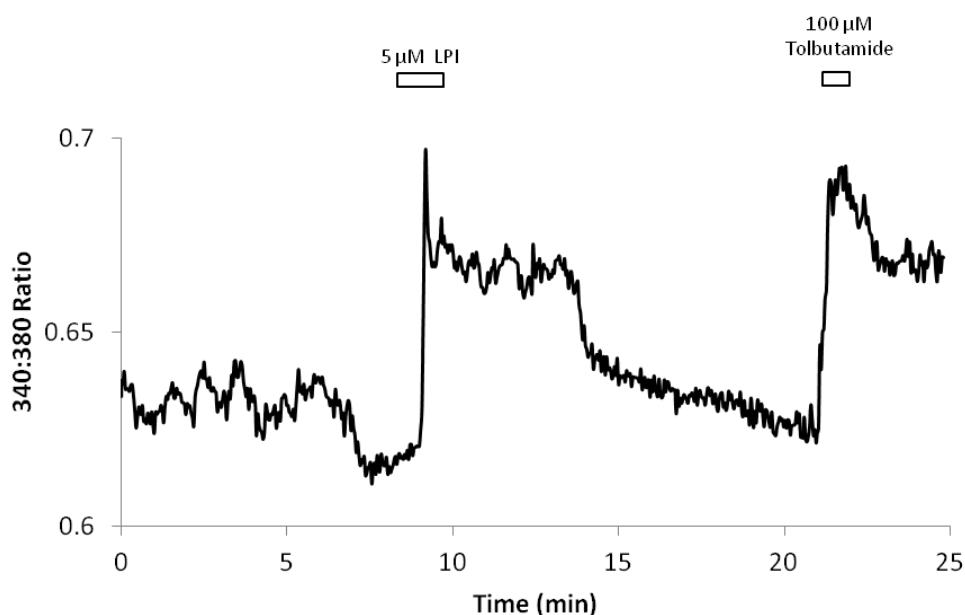
A Dispersed human islet cells with O-1602**B** Dispersed human islet cells with LPI

Figure 4.3.1.1 Effect of O-1602 and LPI on $[Ca^{2+}]_i$ in dispersed human islet cells.

Fura-2-loaded dispersed human islet cells were perfused with physiological buffer containing 2mM glucose and supplemented with O-1602 (**A**) and LPI (**B**) as shown. Changes in $[Ca^{2+}]_i$ were measured by single-cell calcium microfluorimetry and expressed as a ratio of 340nm/380nm fluorescence. Results shown here are single cell traces representative of at least 12 cells within a single field from 2 different experiments. 100μM tolbutamide was used at the end of each experiment as a positive control.

4.3.4 Effect of O-1602 on RhoA small GTPase activation in MIN6 cells.

Data presented so far have demonstrated that O-1602 is able to act via GPR55 to trigger increases in $[Ca^{2+}]_i$ in islet cells, possibly by activating the Gq subunit. Other studies have suggested that GPR55 can also be coupled to the G12/13 subunit, which activates a class of second messenger known as Rho small GTPases. To determine if this is the case in islet cells, activities of RhoA, a key Rho small GTPase activated by GPR55 in other cells (Henstridge et al., 2009a; Obara et al., 2011; Ryberg et al., 2007; Whyte et al., 2009), were measured in serum-starved MIN6 cells after exposure to buffers supplemented with 20mM glucose and 10 μ M O-1602. As shown in Figure 4.3.4.1, MIN6 cells maintained overnight in low glucose (5mM) and serum-free conditions expressed RhoA, but there was very low detectable RhoA activity. Exposure of MIN6 cells to 20mM glucose for 10 minutes induced RhoA activation, which was detected by immunoblotting after the pull-down assay described in Section 2.6.2 (8.3 ± 3.7 fold increase in RhoA activity, mean \pm SEM, n=3). Parallel experiments showed that 10 μ M O-1602 failed to elicit RhoA activation at 2mM glucose, and, then at 20mM glucose, Figure 4.3.4.1B indicated that there was no significant change in fold increase of RhoA activity in response to O-1602.

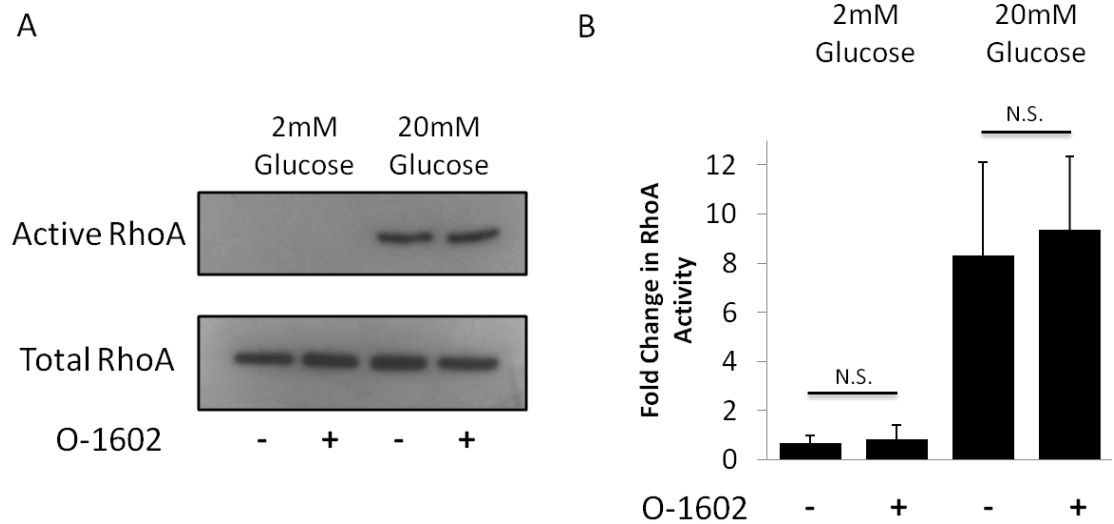


Figure 4.3.4.1 O-1602 does not promote RhoA activation in MIN6 cells.

MIN6 cells grown in low glucose (5mM) and serum-free conditions overnight were incubated with physiological buffer containing 2mM or 20mM glucose supplemented with or without 10 μ M O-1602. Immunoblot (**A**) and fold change in RhoA activity relative to control (**B**) illustrated that 20mM glucose induced RhoA activation in MIN6 cells. No effect was observed when MIN6 cells were treated with O-1602 at 2mM glucose and 20mM glucose. Data is presented as mean \pm SEM, n=3.

4.4 Discussion

Research in other cell types has suggested that GPR55 is coupled to either Gq or G12/13 α subunits (Henstridge et al., 2009a; Lauckner et al., 2008; Ryberg et al., 2007). Activated Gq recruits phospholipase C β (PLC β) to the cell membrane and activated PLC β which then converts phosphatidylinositol 4,5-bisphosphate (PIP₂) to inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ diffuses into the cytosol and activates IP₃ receptors to release Ca²⁺ from the endoplasmic reticulum. It has been reported that pharmacological activation of GPR55 resulted in elevations of [Ca²⁺]_i (Sylantsev et al., 2013; Waldeck-Weiermair et al., 2008). One study working with rat islets reported that O-1602 potentiated glucose-induced increase in [Ca²⁺]_i (Romero-Zerbo et al., 2011). An increase in [Ca²⁺]_i is responsible for regulating a variety of islet functions such as hormone secretion and cell survival. On the other hand, the G12/13 α subunit is linked to activation of Rho small GTPases and RhoA was suggested as the key GTPase activated by GPR55 ligands. However, RhoA and its downstream kinase ROCK were demonstrated to have an inhibitory effect on insulin secretion (Hammar et al., 2009; Tomas et al., 2010). It is important to determine which pathway is activated by GPR55 in order to better understand its physiological roles in islets.

The previous chapter has shown that GPR55 is expressed at both mRNA and protein levels in MIN6 cells, and in mouse and human islets. Calcium microfluorimetry data herein have demonstrated that the GPR55 agonist O-1602, the putative endogenous ligand LPI and the GPR55 antagonist CBD all stimulated increases in [Ca²⁺]_i in MIN6 cells and mouse β -cells. In addition, O-1602 and LPI also caused elevations in [Ca²⁺]_i in dispersed human islet cells. When comparing the effects of these compounds on calcium handling by β -cells from WT and GPR55 KO mice, it was shown that O-1602 required expression of the GPR55 receptor to stimulate [Ca²⁺]_i, while LPI and CBD acted independently of GPR55.

The ability of O-1602 to increase $[Ca^{2+}]_i$ by O-1602 has been demonstrated in a variety of cell types (Sylantsev *et al.*, 2013; Waldeck-Weiermair *et al.*, 2008), including islet β -cells (McKillop *et al.*, 2013; Romero-Zerbo *et al.*, 2011). It was shown that O-1602 stimulated increases in $[Ca^{2+}]_i$ in rat insulin-secreting cell line BRIN-BD11 cells at both 5.6mM and 16.7mM glucose (McKillop *et al.*, 2013). On the other hand, another study reported that O-1602 had no effect on calcium oscillations in rat islets at 2mM glucose, but it potentiated glucose-induced increase in $[Ca^{2+}]_i$ (Romero-Zerbo *et al.*, 2011). Data presented in this chapter are consistent with findings in BRIN-BD11 cells, which showed that O-1602 increased $[Ca^{2+}]_i$ in islet cells at both sub-stimulatory and stimulatory concentrations of glucose. Elevations in $[Ca^{2+}]_i$ at 2mM glucose were not reported in rat islets, possibly due to whole rat islets were used for calcium measurement in the previous publication. It is interesting to observe that only approximately 50% and 40% of β -cells (those that were glucose/CCh/tolbutamide-responsive) responded to O-1602 treatment at 2mM and 20mM glucose, respectively. This suggests functional heterogeneity among islet β -cells and it has been reported before that islet β -cells are different in their $[Ca^{2+}]_i$ responses (Squires *et al.*, 2000; Squires *et al.*, 2002). However, it is unclear why a smaller percentage of β -cells responded to O-1602 at 2mM glucose, than at 20mM glucose. In order to further investigate if O-1602 interacted with GPR55 to mediate its effect on $[Ca^{2+}]_i$ in islet cells, calcium handling by islet cells from GPR55 KO mice was also investigated. Less than 10% GPR55 null β -cells responded to O-1602 at 2mM glucose and none of them responded at 20mM glucose. This suggested that O-1602 signals mainly through GPR55 at a sub-stimulatory concentration of glucose and acts specifically via GPR55 at a maximum stimulatory concentration of glucose to manipulate changes in $[Ca^{2+}]_i$ in mouse islet cells. This agrees with other studies describing O-1602 as a specific GPR55 agonist (Lauckner *et al.*, 2008; Waldeck-Weiermair *et al.*, 2008). On the other hand, O-1602 may potentially interact with other cell surface proteins in islet cells isolated from GPR55 KO mice and it was recently shown that O-1602 was able to trigger migration of HEK293 cells overexpressing the GPR18 receptor (McHugh *et al.*, 2010). However, a recent study by our group did not detect

GPR18 mRNA expression in human islets (Amisten *et al.*, 2013) and GPR18 expression by mouse islets has not been investigated to date.

LPI is believed by some researchers to interact specifically with GPR55 to mediate changes in $[Ca^{2+}]_i$ (Oka *et al.*, 2007; Pineiro *et al.*, 2011; Sylantsev *et al.*, 2013; Waldeck-Weiermair *et al.*, 2008), but GPR55-independent mobilisation of intracellular calcium by LPI were also reported (Bondarenko *et al.*, 2010; Bondarenko *et al.*, 2011a, b). The ability of LPI to stimulate elevations in $[Ca^{2+}]_i$ in islets has been demonstrated previously (Metz, 1988; Rustenbeck and Lenzen, 1992), but no studies have addressed whether LPI acts specifically on GPR55 to mediate these effects. Experiments presented in this chapter also showed that LPI was able to induce elevations of $[Ca^{2+}]_i$ in MIN6 cells, mouse β -cells and dispersed human islet cells. More mouse β -cells responded to LPI (Figure 3.4.2.2E) at both 2mM and 20mM glucose in comparison to O-1602 (Figure 4.3.2.1E). However, parallel experiments using islet cells from GPR55 KO mice demonstrated that a similar number of β -cells from GPR55 KO mice responded to LPI with increases in $[Ca^{2+}]_i$ at 2mM and 20mM glucose as has been observed with WT islets. This finding is in line with previous research showing that LPI increased $[Ca^{2+}]_i$ independently from GPR55 activation (Bondarenko *et al.*, 2010; Bondarenko *et al.*, 2011a, b). It is not clear how LPI elevates $[Ca^{2+}]_i$ in β -cells, but it has been suggested to activate cation channels such as the non-selective cation channel, Ca^{2+} -activated K^+ channel and L-type voltage-gated calcium channel (Ben-Zeev *et al.*, 2010; Bondarenko *et al.*, 2010; Bondarenko *et al.*, 2011a, b). These cation channels are expressed by islet β -cells and play a regulatory role in β -cell electrophysiology (Braun *et al.*, 2008; Ferrer *et al.*, 1996; Leech and Habener, 1998). LPI may interact with them to induce the stimulatory effects observed in this chapter. In addition, LPI has been reported to inhibit Na^+/K^+ ATPase to alter $[Ca^{2+}]_i$ independently of GPR55 (Bondarenko *et al.*, 2010) and it may exert its effects via this mechanism in islets.

CBD has been reported to be an effective GPR55 antagonist (Li *et al.*, 2013; Ryberg *et al.*, 2007; Whyte *et al.*, 2009) and it caused an insignificant calcium increase in HEK293 cells overexpressing GPR55 (Lauckner *et al.*, 2008). However, data presented here have demonstrated that CBD was able to induce elevations in $[Ca^{2+}]_i$ similar to those obtained following exposure to O-1602 and LPI in MIN6 cells at 2mM glucose, and in approximately 50% of mouse β -cells at both 2mM and 20mM glucose. Subsequent experiments investigating β -cells from GPR55 KO mice revealed that CBD did not require GPR55 receptor expression to mediate its effects on increase in $[Ca^{2+}]_i$ in islet cells. Two studies have shown that CBD was able to activate $[Ca^{2+}]_i$ in neuronal cells (Mato *et al.*, 2010; Qin *et al.*, 2008) and one of them suggested that CBD acted via regulating transient receptor potential cation channel subfamily V member 2 (TRPV2) (Qin *et al.*, 2008). It has also been reported that CBD activated peroxisome proliferator-activated receptor γ (PPAR γ) (O'Sullivan *et al.*, 2009) and stimulated PPAR γ thus is known to stimulate insulin secretion from hamster insulinoma cells through K_{ATP} channels and Ca^{2+} influx (Shimomura *et al.*, 2004). The mode of action of CBD in islet cells by CBD remains to be discovered.

The effect of O-1602 on RhoA activation was also investigated in MIN6 β -cells using a commercially available RhoA activation assay kit. Figure 4.3.4.1 has shown that 20mM glucose was able to activate RhoA in MIN6 cells, which are consistent with observations in other studies (Zhang *et al.*, 2012). In contrary with the findings in other cell types (Henstridge *et al.*, 2009a; Obara *et al.*, 2011; Ryberg *et al.*, 2007; Whyte *et al.*, 2009), O-1602 did not induce RhoA activation in MIN6 cells. A 10 minute incubation time was selected for these experiments since it has been demonstrated to be an effective stimulation period for O-1602-induced RhoA activation in other cell lines (Ryberg *et al.*, 2007; Whyte *et al.*, 2009). However, it is possible that RhoA activity may be regulated over a different time course in MIN6 β -cells and further studies are required over a range of incubation periods (e.g. 1, 2, 5, 15 and 30 minutes) for a comprehensive understanding of whether O-1602 regulates the RhoA activity in

β -cells. Since O-1602-induced RhoA activation in islet cells has not been reported previously by the literature, data so far suggests that O-1602-mediated GRP55 activation is not linked to the RhoA-ROCK pathway. Despite that, this cannot exclude that GPR55 is coupled to G 12/13 subunit since one paper has suggested activation of GPR55 also led to upregulations of other Rho small GTPases, Cdc42 and Rac1 (Ryberg *et al.*, 2007).

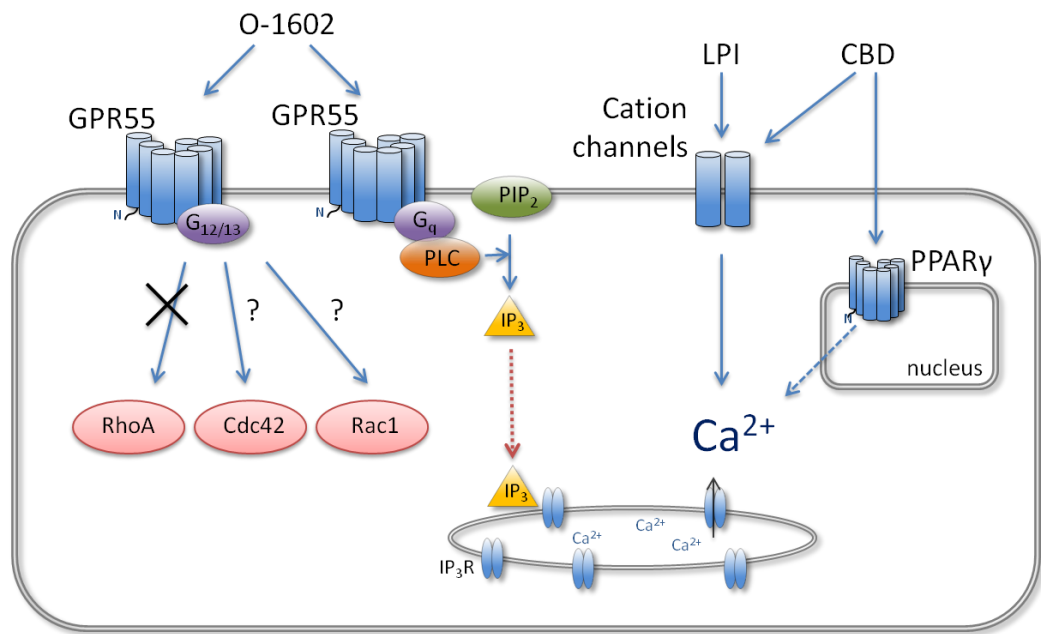


Figure 4.4.1.1 Second messenger generation mediated by O-1602, LPI and CBD.

O-1602 activates GPR55 to induce increase in $[Ca^{2+}]_i$ in β -cells and it does not activate the small GTPase RhoA. It remains unknown whether O-1602 activates other Rho small GTPases such as Cdc42 and Rac1. LPI and CBD also stimulate increases in $[Ca^{2+}]_i$ in β -cells, but these effects are not transduced via GPR55 activation. LPI and CBD may interact with cation channels to mediate their effects and. In addition, CBD is highly membrane permeable (Deiana *et al.*, 2012) and it may also signal through PPAR γ expressed on nuclear membranes.

As described before, Ca^{2+} plays important roles in the regulation of insulin exocytosis during both stages of the biphasic secretory response to glucose and an increase in $[Ca^{2+}]_i$ in β -cells is considered an initiator of insulin release (Antunes *et al.*, 2000; Charles *et al.*, 1975; Henquin, 2000; Howell *et al.*, 1994; Prentki and Matschinsky, 1987b; Squires *et al.*, 2002). On the other

hand, studies have suggested that the RhoA-ROCK pathway plays an inhibitory role in insulin secretion (Hammar *et al.*, 2009; Tomas *et al.*, 2010). Data presented in this chapter demonstrated that O-1602 activates GPR55 to stimulate increase in $[Ca^{2+}]_i$ in islet cells and it does not activate the RhoA small GTPase in MIN6 cells as illustrated in Figure 4.4.1.1. CBD and LPI also activate $[Ca^{2+}]_i$, but their actions are independent of GPR55. These observations lead to a hypothesis that O-1602, LPI and CBD may stimulate insulin secretion and the next chapter will investigate the effects of these ligands on insulin secretion from mouse and human islets.

Chapter 5 Pharmacological manipulation of GPR55 in islets: Insulin secretion

CHAPTER 5 PHARMACOLOGICAL MANIPULATION OF GPR55 IN ISLETS:**INSULIN SECRETION****5.1 Introduction**

There is good evidence that the CB1 and CB2 cannabinoid receptors play an important role in regulating hormone secretion from islets of Langerhans (Di Marzo, 2008; Li et al., 2011b). Perfusion experiments carried out by our group and experiments by others have shown that activation of CB1 and CB2 stimulated insulin secretion (Li et al., 2011a; Li et al., 2010; Vilches-Flores et al., 2010), while a reduction in insulin output from islets following pharmacological activation of cannabinoid receptors has been demonstrated by other studies using static incubation experiments (Juan-Pico et al., 2006; Nakata and Yada, 2008). The differences in the reported effects of cannabinoids on insulin secretion may be a consequence of the model used, since a comparison of the effects of the endocannabinoid anandamide in rat islets indicated that it stimulated insulin secretion from islets that had been maintained in culture overnight, while it exerted inhibitory effects on freshly isolated islets (Anderson *et al.*, 2013).

The effects of GPR55 on islet hormone secretion were unknown until recent studies, published during the course of this thesis, demonstrated that activation of GPR55 by pharmacological agonists stimulated insulin secretion from BRIN-BD11 insulin-secreting cells and potentiated glucose-induced insulin secretion from rat and mouse islets (McKillop *et al.*, 2013).

A stimulatory effect of LPI on insulin secretion was first reported in a 1986 study, which indicated that this phospholipid induced a concentration-dependent, reversible increase in insulin secretion from rat islets (Metz, 1986), and a subsequent study by the same author reported that LPI was able to elevate $[Ca^{2+}]_i$ in whole rat islets (Metz, 1988). A more recent

study demonstrated that LPI stimulated intracellular cAMP accumulation in a concentration-dependent manner in the NIT-1 β -cell line (Soga *et al.*, 2005). Elevations in intracellular cAMP may indicate that LPI signals through a receptor coupled to the Gs α subunit. Alternatively, the increases in cAMP may be secondary to activation of Ca^{2+} -sensitive isoforms of adenylate cyclase as a consequence of LPI-induced elevation of $[\text{Ca}^{2+}]_i$, both of which would result in enhanced insulin secretion.

Only in the last few years has the revelation that LPI may act as a GPR55 endogenous agonist (Henstridge *et al.*, 2009a; Lauckner *et al.*, 2008; Oka *et al.*, 2007; Oka *et al.*, 2009; Waldeck-Weiermair *et al.*, 2008) provided new insight into the possible mode of action of LPI in regulating insulin secretion. It has been reported that GPR55 couples to either Gq or G12/13 α subunits (Ross, 2009; Sharir and Abood, 2010). The lack of interaction with G_s indicates that if LPI induces cAMP accumulation through GPR55 activation this does not occur through direct G-protein coupling. In addition, calcium microfluorimetry experiments in the last chapter have shown that LPI-induced increases in β -cell $[\text{Ca}^{2+}]_i$ were not dependent on GPR55, but the exact mechanism of action responsible for LPI-induced effects in islets remains to be discovered.

Experiments in the previous chapter demonstrated that O-1602 and LPI stimulated increases in $[\text{Ca}^{2+}]_i$ in MIN6 β -cells, and in dispersed mouse and human islet cells. In addition, studies using islets from GPR55 KO mice illustrated that the effects of O-1602, but not LPI, were dependent on GPR55 expression. The GPR55 antagonist CBD unexpectedly had stimulatory effects on $[\text{Ca}^{2+}]_i$ in mouse β -cells, and, similar to LPI, the stimulatory effects of CBD were shown to be GPR55-independent. The experiments described in this chapter investigated whether O-1602, LPI and CBD are able to stimulate insulin secretion from mouse and human islets, and studies using islets isolated from WT and GPR55 KO C57/BL6 mice further examined if these effects were GPR55-dependent. A perfusion system was used to measure

dynamic insulin secretion from islets, since it is more sensitive to detect small changes in hormone secretion than static incubation experiments.

5.2 Methods

5.2.1 Measurement of insulin secretion from perfused isolated mouse and human islets

Mouse islets were isolated from either ICR or C57/BL6 mice as described in Section 2.2.1 and human islets were obtained from the King's College London Human Islet Isolation Unit with appropriate ethical approval (Section 2.2.2). For dynamic measurements of insulin secretion using the perfusion system, 40-50 mouse or human islets were transferred to chambers containing 1µm pore-size nylon filters and perfused at a flow rate of 0.5ml/ml with physiological buffers (Table 2.7.1.1) containing 2mM or 20mM glucose in the absence or presence of ligands of interest (10µM O-1602, 5µM LPI or 1µM CBD) in a temperature-controlled environment (37°C) (Section 2.7.1). Medium containing insulin secreted from islets in the chambers was collected every 2 minutes and insulin was quantified by radioimmunoassay (Section 2.7.2).

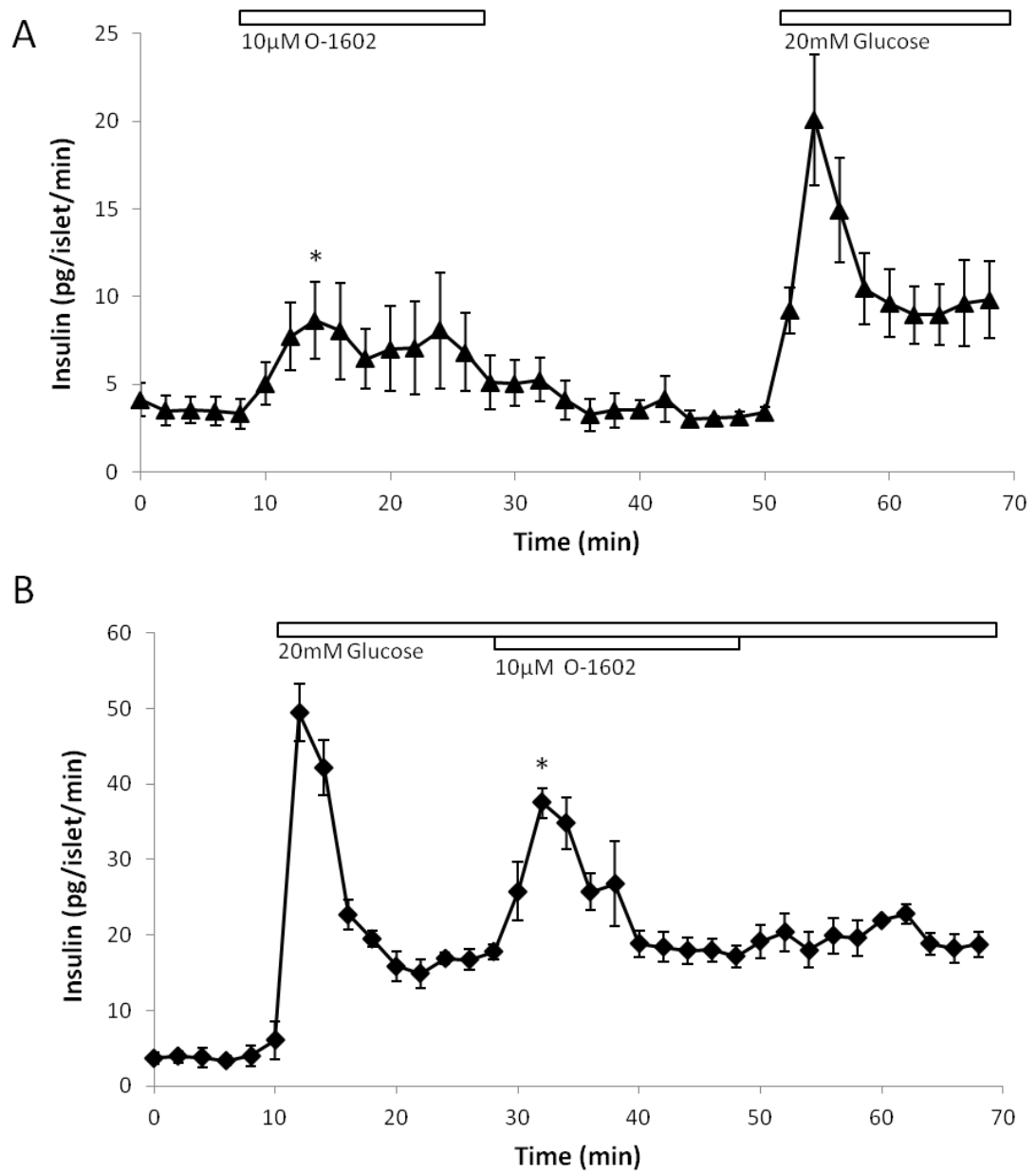
5.3 Results

5.3.1 Effects of O-1602 on insulin secretion from mouse islets

The effects of O-1602 on insulin secretion were initially studied using islets from ICR mice, as this is the strain of mice used by the islet biology group at King's College London for determining insulin secretagogue effects of ligands of interest. 10 μ M of O-1602 was used since data presented in the previous chapter indicated that it was able to induce elevations in islet β -cell $[Ca^{2+}]_i$. Figure 5.3.1.1A shows that O-1602 induced a small, but significant increase in insulin secretion from isolated, perfused ICR mouse islets at 2mM glucose. Addition of 10 μ M O-1602 caused an approximately two-fold increase in insulin release, which was abolished following its removal. After exposure to O-1602 the islets responded further to 20mM glucose with a biphasic increase in insulin secretion that peaked at approximately six-fold higher than secretion at 2mM glucose. Figure 5.3.1.1B shows that O-1602 also potentiated glucose-induced insulin secretion from mouse islets. A rapid elevation in insulin secretion was caused by 20mM glucose and addition of 10 μ M O-1602 during the second phase induced a short-lived increase in insulin secretion. This response first peaked at approximately two-fold higher than the 20mM glucose plateau and returned back to the second phase of glucose-induced secretion before the withdrawal of O-1602.

To assess whether the effects of O-1602 were mediated via activation of GPR55, perfusion experiments were performed using islets isolated from WT and GPR55 KO C57/BL6 mice. Consistent with the data presented in Figure 5.3.1.1A, islets from WT C57/BL6 mice responded to the challenge of 10 μ M O-1602 with a small, prolonged increase in insulin secretion at 2mM glucose. This stimulatory effect of O-1602 on basal insulin secretion was absent in islets isolated from GPR55 KO mice (Figure 5.3.1.1C). Both groups of islets were then perfused with buffer supplemented with 20mM glucose followed by administration of 10 μ M O-1602. 20mM glucose induced a rapid, biphasic increase in insulin release from both WT and GPR55 null mouse islets. O-1602 was able to potentiate glucose-induced insulin

secretion from WT mouse islets to two-fold higher than the 20mM glucose plateau, but it was longer-lived compared to the transient response obtained with islets from ICR mice (Figure 5.3.1.1B). In contrast, the effects of O-1602 on glucose-induced insulin secretion from GPR55 KO islets were significantly impaired compared to the response observed in WT islets (Figure 5.3.1.1C).



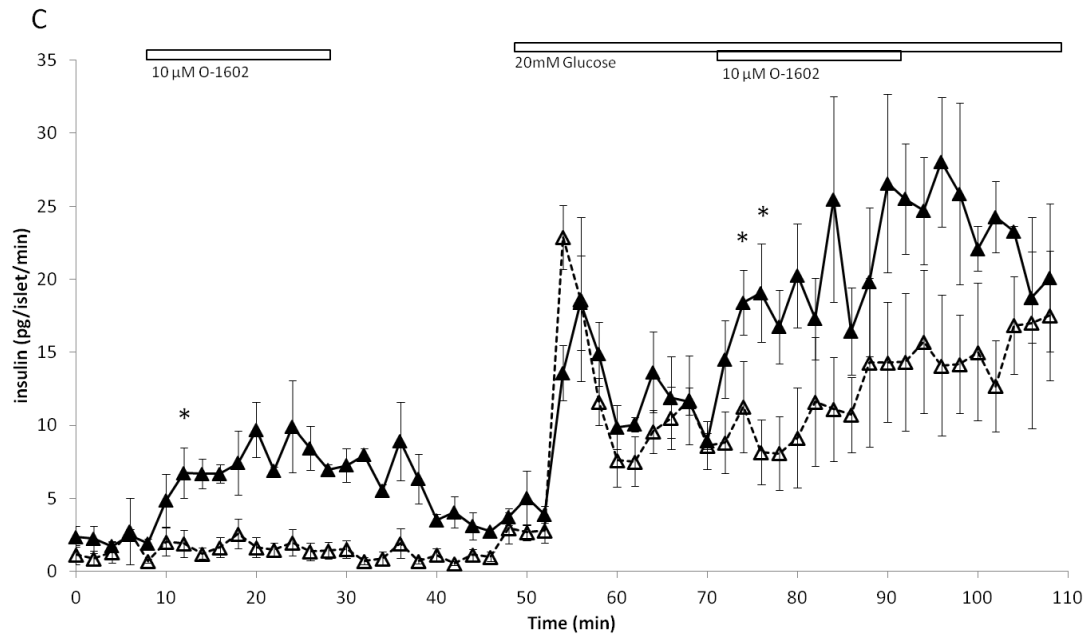


Figure 5.3.1.1 O-1602 stimulates insulin secretion from mouse islets through GPR55 activation.

Islets isolated from ICR mice (panels A and B) and C57/BL6 WT or GPR55 KO mice (panel C) were perfused with physiological buffer containing 2mM glucose and supplemented as shown. **A** Addition of 10μM O-1602 at 2mM glucose resulted in a two-fold, reversible increase in insulin secretion and these islets further responded to 20mM glucose with a biphasic stimulation of insulin secretion. **B** 10μM O-1602 induced a transient potentiation of glucose-induced insulin secretion. **C** O-1602 also stimulated basal and glucose-induced insulin secretion from islets isolated from WT C57/BL6 mice (black triangle). Parallel experiments using islets isolated from GPR55 KO mice showed that the effects of O-1602 were abolished at 2mM glucose and significantly reduced at 20mM glucose (white triangle). Data are representative of three separate experiments and expressed as means \pm SEM, $n=4$. * $p < 0.05$, peak stimulation vs. basal.

5.3.2 Effects of O-1602 on insulin secretion from human islets

Perfusion experiments were also performed to investigate the effects of O-1602 on insulin secretion from isolated human islets, and these indicated that this GPR55 agonist also had stimulatory effects on human β -cells. Thus, it can be seen from Figure 5.3.2.1A that 10 μ M O-1602 caused a biphasic elevation in insulin secretion from perfused, isolated human islets at 2mM glucose. Due to the difficulties in obtaining purified human islets, free from contaminating exocrine tissues, it cannot be guaranteed that all samples loaded into the perfusion chambers were human islets. Therefore the data here have been presented as percentage of insulin secretion at 2mM glucose rather than pg insulin/islet/min, which was used for mouse islet perfusion studies where highly purified mouse islet preparations were used. The insulin secretory response reached a five-fold peak stimulation within 8 minutes exposure to O-1602, which was greater than the O-1602-induced peak stimulation from mouse islets. Insulin release then reached a plateau at approximately two-fold basal in the continued presence of O-1602, and this was readily reversible upon withdrawal of the agonist. This secretion profile was similar to that obtained with 20mM glucose after the O-1602 treatment in the same group of human islets (Figure 5.3.2.1A). Consistent with the finding from perfusion experiments with mouse islets (Figure 5.3.1.1B), 10 μ M O-1602 also potentiated, but not significantly, glucose-induced insulin secretion from human islets, with a transient response that peaked approximately two-fold higher than the 20mM glucose plateau (Figure 5.3.2.1B).

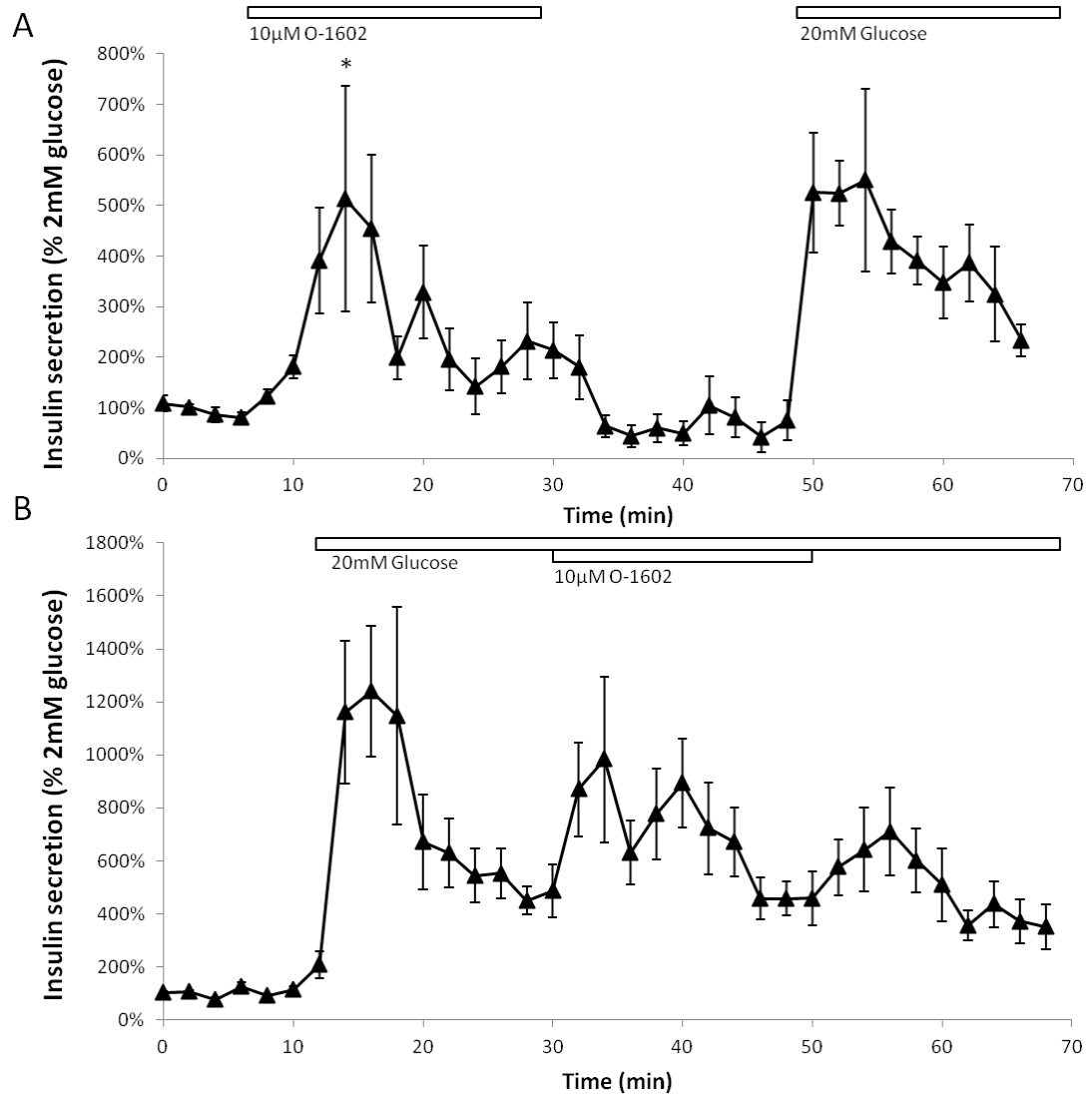


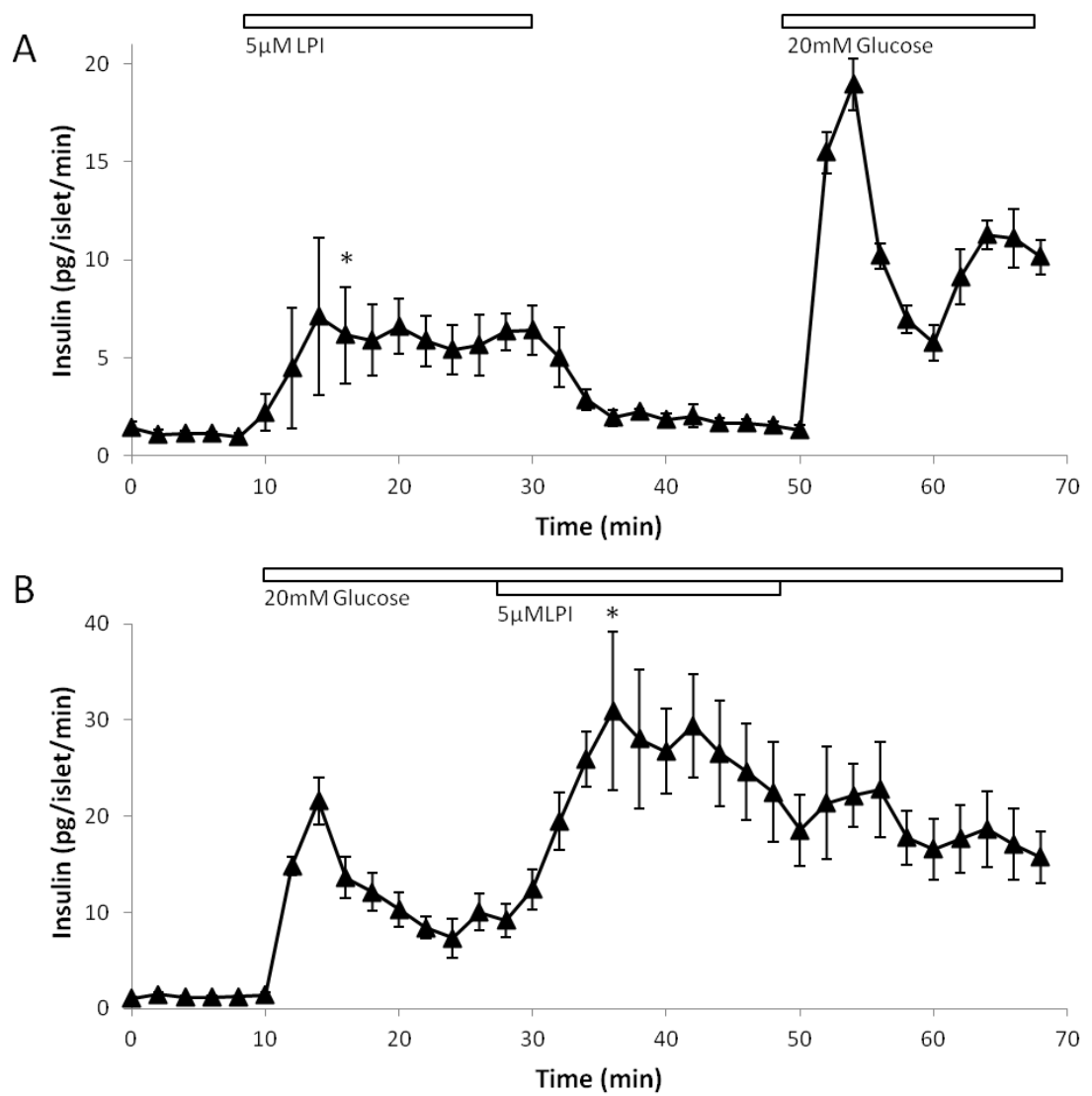
Figure 5.3.2.1 O-1602 stimulates insulin secretion from human islets.

Isolated human islets were perfused with physiological buffer containing 2mM glucose and supplemented as shown. **A** Addition of 10µM O-1602 at 2mM glucose resulted a two-fold, reversible increase in insulin secretion from perfused isolated human islets, which further responded to 20mM glucose with a biphasic stimulation of insulin secretion. **B** 10µM O-1602 induced a transient, but not significant potentiation of glucose-induced insulin secretion. Data are representative of three separate experiments using islets from different donors and are expressed as means \pm SEM, $n=4$. * $p < 0.05$, peak stimulation vs. basal.

5.3.3 Effects of LPI on insulin secretion from mouse islets

The stimulatory effect of LPI on insulin secretion from rat islets was first reported nearly thirty years ago (Metz, 1986), but it was only much more recently that LPI was proposed as an endogenous ligand of GPR55. Perfusion experiments with mouse islets isolated from ICR mice confirmed the earlier report of stimulation of insulin secretion by exogenous LPI, with the demonstration that 5 μ M LPI caused a sustained elevation of insulin release that was approximately five-fold higher than basal secretion at 2mM glucose (Figure 5.3.3.1A). The maintained response to LPI was similar to that obtained with O-1602 at 2mM glucose (Figure 5.3.1.1A and C). In addition, LPI enhanced glucose-induced insulin secretion, inducing a sustained, three-fold potentiation after mouse islets had responded to 20mM glucose with a biphasic insulin secretion profile (Figure 5.3.3.1B).

Perfusions were also performed using islets isolated from GPR55 KO mice to determine whether the stimulatory effects of LPI were dependent on GPR55. When investigating the effects of 5 μ M LPI using WT C57/BL6 mouse islets in several separate experiments it was observed that there was only a transient, non-significant elevation of insulin release at 2mM glucose (Figure 5.3.3.1C), in contrast to the large, sustained response to LPI obtained with ICR mouse islets (Figure 5.3.3.1A). Nonetheless, LPI stimulated a sustained potentiation of glucose-induced insulin secretion from C57/BL6 islets (Figure 5.3.3.1C), similar to the effect seen in ICR islets (Figure 5.3.3.1B). This potentiation of insulin release by LPI was independent of GPR55 activation since the same stimulatory profiles of LPI were observed in mouse islets obtained from both WT and GPR55 KO mice.



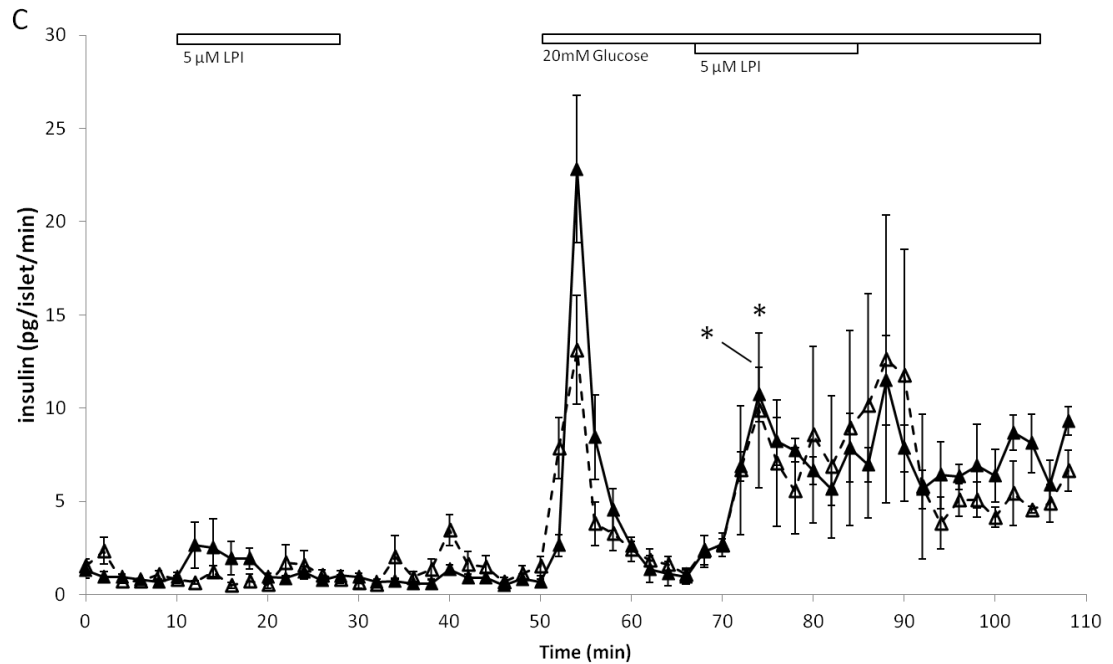


Figure 5.3.3.1 LPI stimulates insulin secretion from mouse islets, independent of GPR55 receptor activation.

Islets isolated from ICR mice (panels A and B) and C57/BL6 WT or GPR55 KO mice (panel C) were perfused with physiological buffer containing 2mM glucose and supplemented as shown. **A** Addition of 5μM O-LPI at 2mM glucose resulted in a five-fold, reversible increase in insulin secretion and these islets further responded to 20mM glucose with a biphasic stimulation of insulin secretion. **B** 5μM LPI induced a sustained potentiation of glucose-induced insulin secretion. **C** LPI failed to significantly stimulate insulin release at 2mM glucose, but it induced a two-fold potentiation of glucose-induced insulin secretion from islets isolated from WT C57/BL6 mice (black triangle). LPI also potentiated secretion in parallel experiments using islets isolated from GPR55 KO mice (white triangle). Data are representative of three separate experiments and expressed as means \pm SEM, $n=4$. * $p < 0.05$, peak stimulation vs. basal.

5.3.4 Effects of LPI on insulin secretion from human islets

A further series of perfusion experiments was conducted to investigate the effects of LPI on insulin secretion from isolated human islets. The addition of 5 μ M LPI at 2mM glucose resulted in a biphasic insulin secretory response (Figure 5.3.4.1A), similar to O-1602-induced insulin secretion from human islets at 2mM glucose (Figure 5.3.2.1A). Insulin secretion after the addition of LPI reached a maximum ten-fold stimulation over basal and it returned to a level about two-fold higher than basal before the removal of LPI. The LPI-induced insulin secretion profile was similar to that of the glucose-induced response when the same groups of human islets were treated with 20mM glucose after exposure to LPI. LPI also caused a large, sustained potentiation of glucose-induced insulin secretion from perfused human islets (Figure 5.3.4.1B), which was consistent with the profile obtained with ICR mouse islets (Figure 5.3.3.1B).

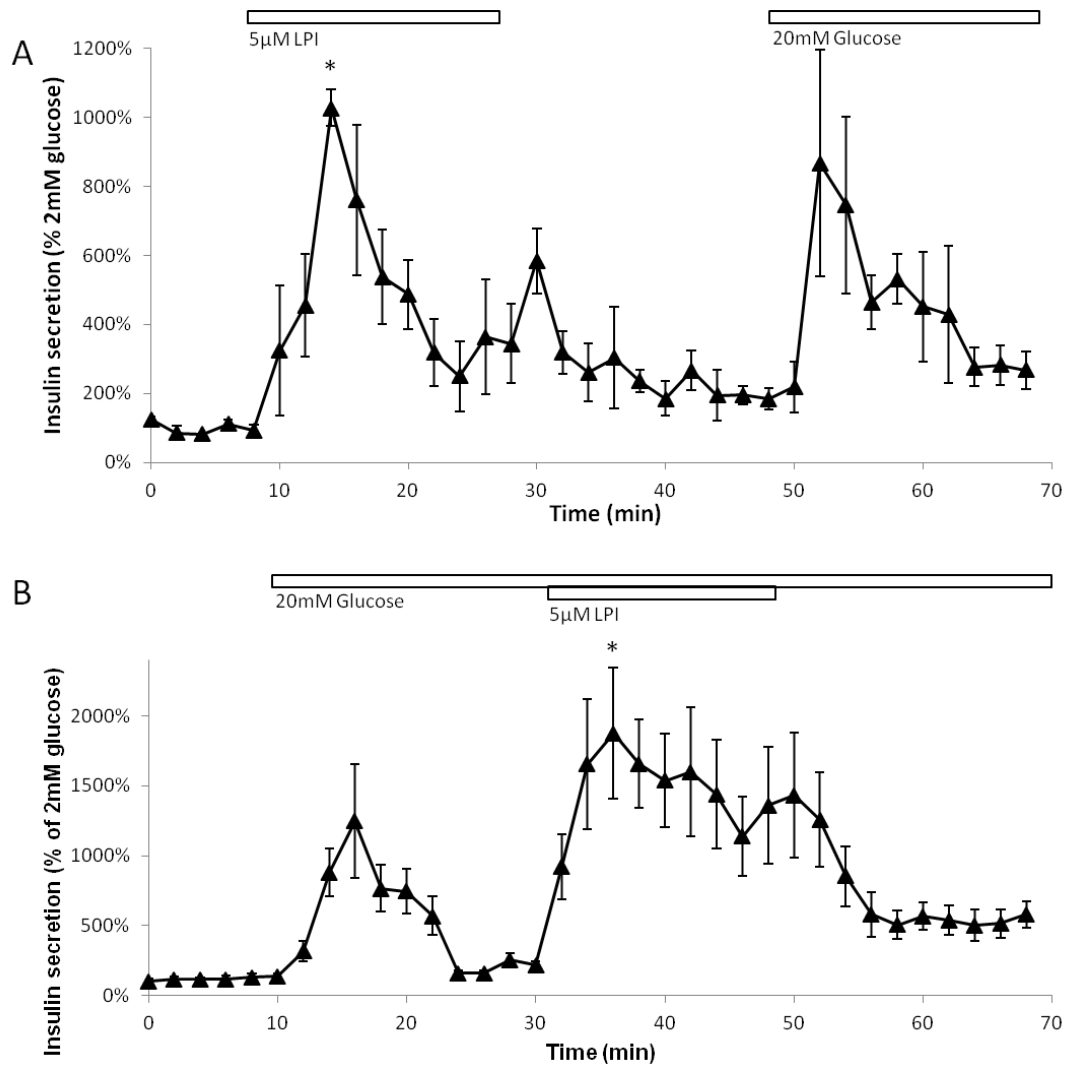


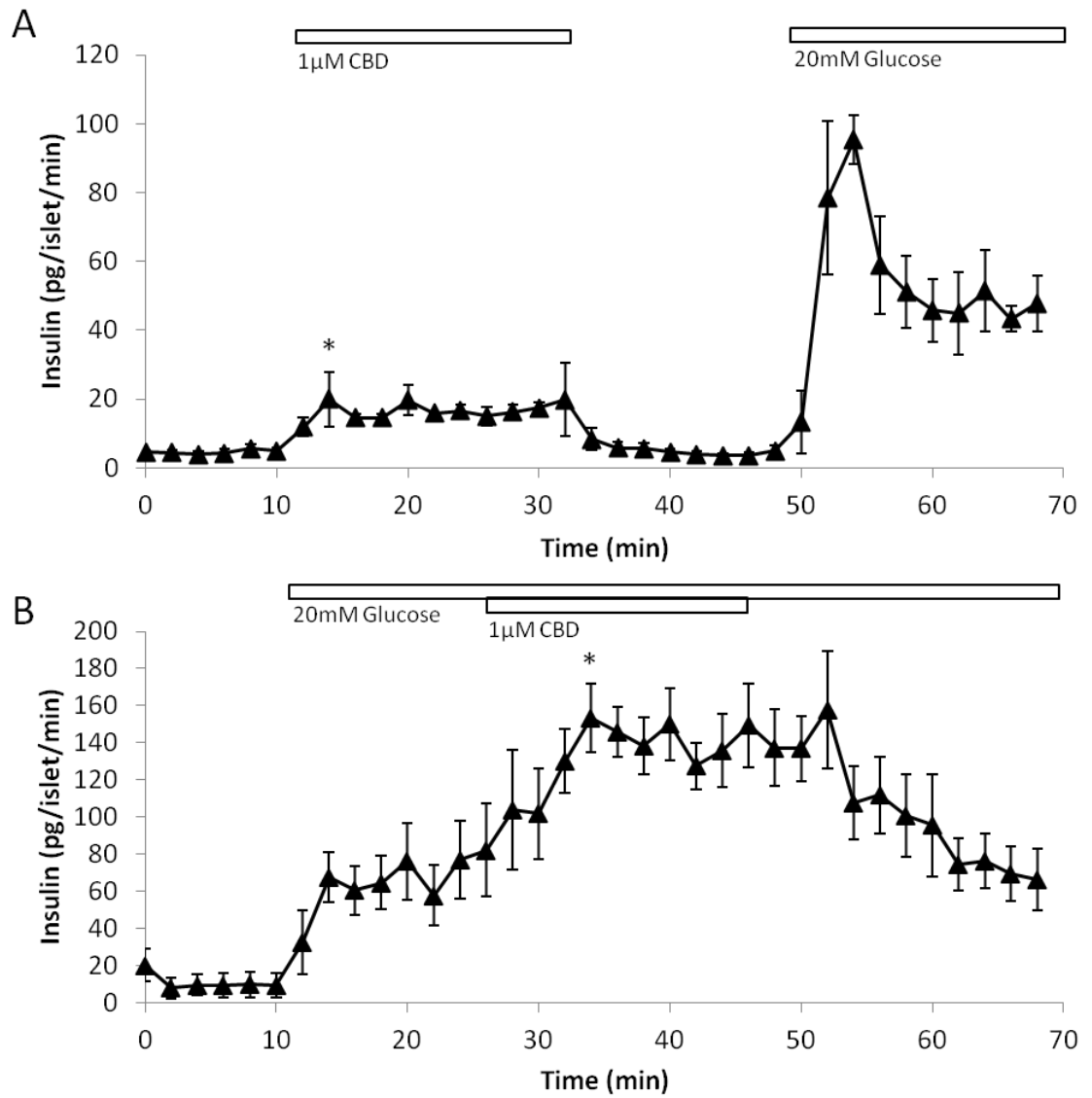
Figure 5.3.4.1 LPI stimulates insulin secretion from human islets.

Isolated human islets were perfused with physiological buffer containing 2mM glucose and supplemented as shown. **A** Addition of 5μM LPI at 2mM glucose resulted a biphasic stimulation of insulin secretion from perfused isolated human islets, which further responded to 20mM glucose with a biphasic stimulation of insulin secretion. **B** 5μM LPI induced an approximately eight-fold, sustained enhancement of the second phase of glucose-induced insulin secretion. Data are representative of three separate experiments using islets from different donors and are expressed as means \pm SEM, $n=4$. * $p < 0.05$, peak stimulation vs. basal.

5.3.5 Effects of CBD on insulin secretion from mouse islets.

The effects of the GPR55 antagonist CBD on insulin secretion were also studied. Data presented in the last chapter indicated that CBD did not show antagonist activity on β -cell $[Ca^{2+}]_i$. Instead, it stimulated elevations in $[Ca^{2+}]_i$ in MIN6 cells and mouse islets, effects that were maintained in GPR55 null islets. Consistent with this agonist effect of CBD, perfusion experiments using islets isolated from ICR mice demonstrated that 1 μ M CBD caused an approximately four-fold sustained increase in insulin secretion at 2mM glucose (Figure 5.3.5.1A), larger in amplitude than the effect obtained with the pharmacological GPR55 agonist O-1602. When ICR islets were exposed to 1 μ M CBD after 20mM glucose, CBD potentiated the glucose-induced secretory response to approximately two-fold higher than the second phase plateau (Figure 5.3.5.1B). The effects seen with CBD were similar in magnitude and duration to those observed with LPI.

Investigation of the requirement of GPR55 for the stimulatory effects of CBD on insulin secretion was carried out in perfusion experiments, comparing responses of islets isolated from WT and GPR55 KO C57/BL6 mice. In line with the observations made with ICR mouse islets (Figure 5.3.5.1A and B), 1 μ M CBD induced insulin secretion at 2mM glucose and potentiated glucose-induced insulin secretion from islets isolated from WT mice (Figure 5.3.5.1C). However, the stimulatory effects of CBD at 2mM glucose were not significant in islets from both WT and GPR55 KO mice. In addition, CBD induced an transient effect at 20mM glucose in C57/BL6 islets, in contrast to its prolonged effect in islets from ICR mice. The stimulation of insulin secretion by CBD was also seen in islets isolated from GPR55 KO mice. Thus, although glucose-induced insulin secretion from GPR55 KO islets peaked at a lower level than that from WT islets, 1 μ M CBD was still able to induce a potentiation with a similar magnitude, and its secretagogue effects at 2mM glucose were also preserved (Figure 5.3.5.1C).



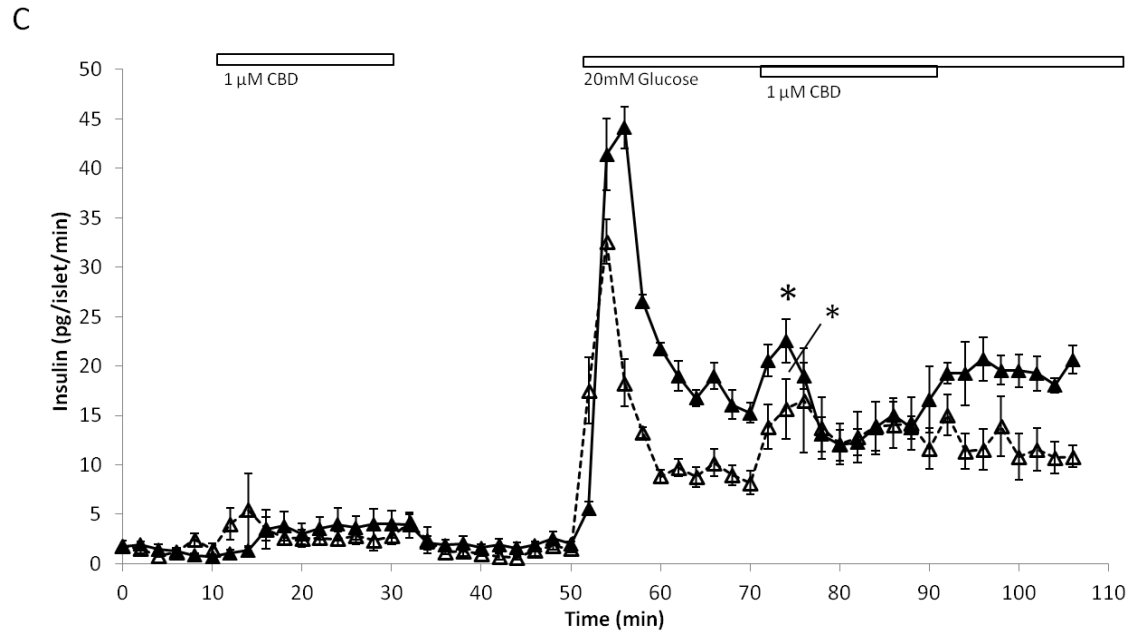


Figure 5.3.5.1 CBD stimulates insulin secretion from mouse islets, independent of GPR55 receptor activation.

Islets isolated from ICR mice (panels A and B) and C57/BL6 WT or GPR55 KO mice (panel C) were perfused with physiological buffer containing 2mM glucose and supplemented as shown. **A** Addition of 1 μ M CBD at 2mM glucose resulted in a four-fold, reversible increase in insulin secretion and these islets further responded to 20mM glucose with a biphasic stimulation of insulin secretion. **B** 1 μ M CBD induced a sustained potentiation of glucose-induced insulin secretion. **C** CBD also stimulated basal (not significant) and glucose-induced insulin secretion from islets isolated from WT C57/BL6 mice (black triangle), and these effects were preserved in parallel experiments using islets isolated from GPR55 KO mice (white triangle). Data are representative of three separate experiments and expressed as means \pm SEM, n=4. * p < 0.05, peak stimulation vs. basal.

5.3.6 Effects of CBD on insulin secretion from human islets

Stimulatory effects of 1 μ M CBD on basal and glucose-stimulated insulin secretion were also seen in experiments using perfused human islets. (Figure 5.3.6.1). However, in contrast to the sustained stimulation seen in mouse islets at 2mM glucose (Figure 5.3.5.1A), CBD-induced insulin secretion from human islets peaked at an approximately four-fold increase above basal and then declined to the pre-stimulatory level before the removal of CBD. A prolonged two-fold potentiation of glucose-induced insulin secretion was recorded when human islets were treated with 1 μ M CBD in the presence of 20mM glucose (Figure 5.3.6.1B).

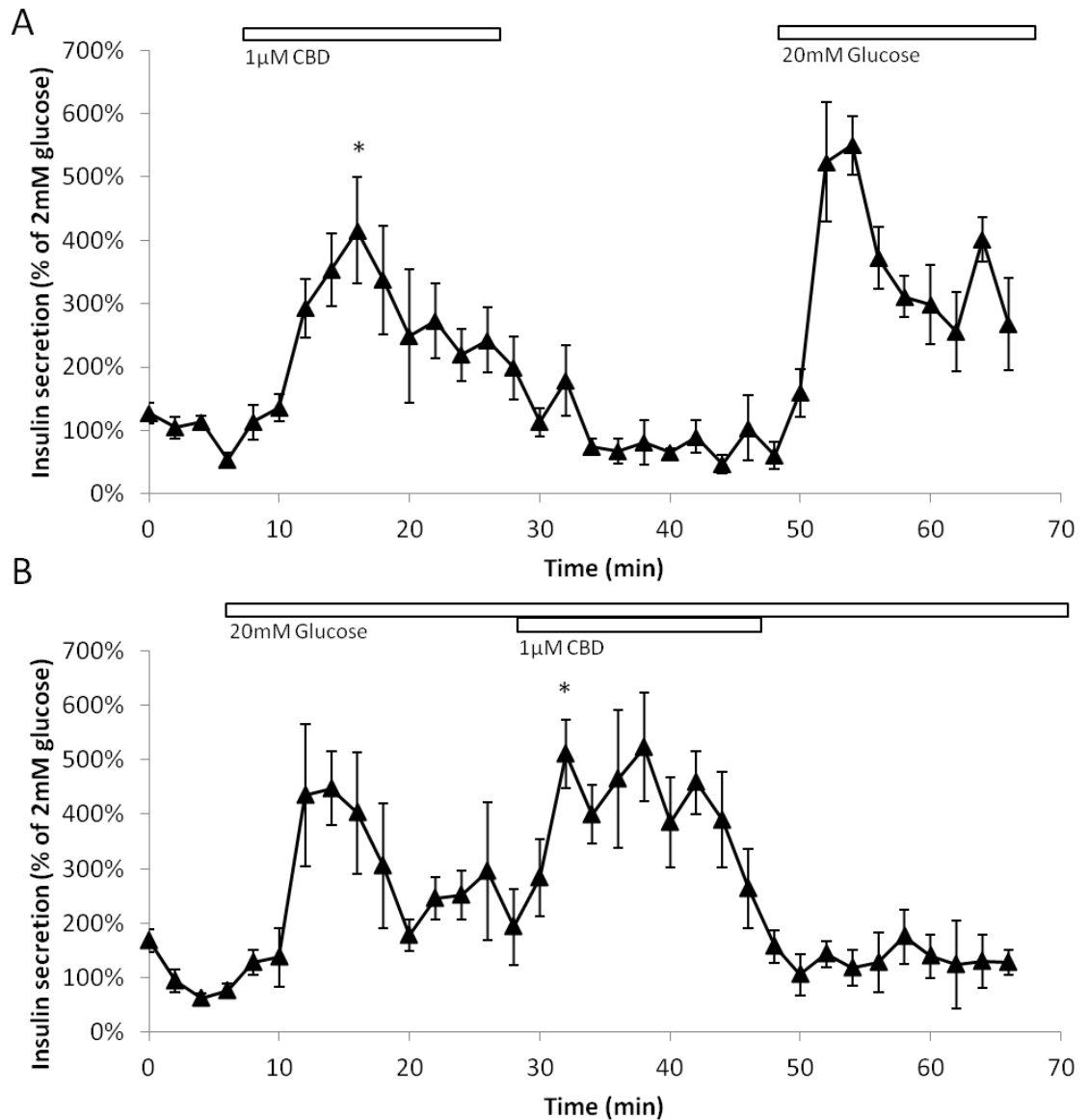


Figure 5.3.6.1 CBD stimulates insulin secretion from human islets

Isolated human islets were perfused with physiological buffer containing 2mM glucose and supplemented as shown. **A** Addition of 1μM CBD at 2mM glucose resulted a transient stimulation of insulin secretion from perfused isolated human islets, which further responded to 20mM glucose with a biphasic stimulation of insulin secretion. **B** 1μM CBD potentiated glucose-induced insulin secretion. Data are representative of three separate experiments using islets from different donors and are expressed as means \pm SEM, n=4. * $p < 0.05$, peak stimulation vs. basal.

5.4 Discussion

The roles of GPR55 in regulating insulin secretion have only been investigated in the past three years, during the course of the experiments described in this thesis. *In vivo* studies have demonstrated that short-term administration of GPR55 agonists to rats or mice increased plasma insulin levels and improved glucose tolerance (McKillop et al., 2013; Romero-Zerbo et al., 2011). Direct effects of GPR55 agonists at β -cells were reported in a recent study evaluating the effects of various GPR55 ligands on insulin secretion from BR1N-BD11 insulin-secreting cells and mouse islets (McKillop *et al.*, 2013). These experiments indicated that all agonists used - O-1602, AM251, abnormal CBD (a synthetic GPR55-activating CBD analogue) and the phospholipid-derived endocannabinoids OEA and PEA - stimulated insulin secretion. O-1602 has also been reported to stimulate insulin release from rat islets (Romero-Zerbo *et al.*, 2011), and the data shown here extend these observations to human islets, where O-1602 produced significant elevations in insulin output.

The insulin secretion data in response to O-1602 that are presented in this chapter are in agreement with the stimulatory effects of O-1602 on $[Ca^{2+}]_i$ shown in the previous chapter (Section 4.3.3 and 4.3.4) and on insulin secretion from BRIN-BD11 cells and mouse islets reported previously (McKillop *et al.*, 2013). However, the data are not consistent with another publication reporting that 10 μ M O-1602 had no effect on insulin secretion from rat islets at both sub-stimulatory and stimulatory concentrations of glucose (Romero-Zerbo *et al.*, 2011), although stimulation was observed with 0.1 μ M O-1602. The reasons for this discrepancy are not immediately obvious, although Romero-Zerbo and colleagues used static incubations, while the experiments described here were performed in a dynamic perfusion system, which allows assessment of the time-course and reversibility of hormone release. However, this does not fully explain the different outcome of the experiments since static incubations were also carried out in the study by McKillop. A role for GPR55 in mediating the stimulatory effects of

O-1602 was evident from the perfusion experiments carried out using islets from GPR55 KO mice, which demonstrated that O-1602-induced increases in insulin secretion were abolished at 2mM glucose and significantly reduced at 20mM glucose. This is consistent with the observations made in the calcium microfluorimetry experiments (Section 4.3.3) and, collectively, the data suggest that O-1602 acts at GPR55 to exhibit its stimulatory effects in islets. There is the possibility that O-1602 may regulate glucose-induced insulin secretion in part via a GPR55-independent mechanism since it still caused a small and delayed potentiation of glucose-induced insulin secretion in islets from GPR55 KO mice towards the end of the 20 minute treatment period. Full evaluation of the requirement of GPR55 for O-1602-induced potentiation of glucose-stimulated insulin secretion would benefit from perfusion experiments in which islets were exposed to buffers containing 20mM glucose in the absence and presence of O-1602 so that the secretory response to 20mM glucose alone could be determined in the same batches of islets. This would allow evaluation of whether the apparent delayed increase in insulin secretion in from the GPR55 knockout islets after exposure to O-1602 was actually a bona fide response rather than reflecting the profile of the secretory response to 20mM glucose. GPR18 has been suggested to be activated by O-1602 (McHugh *et al.*, 2010), but its expression and roles in mouse islets remain to be investigated.

The pathways through which O-1602 activation of GPR55 causes insulin secretion were not fully examined here, but are likely to be secondary to GPR55-mediated activation of PLC and elevation in $[Ca^{2+}]_i$ and diacylglycerol, both of which are known to be involved in insulin secretion (Jones and Persaud, 2010). In addition, PLC activation can occur by the G13 pathway in addition to conventional Gq coupling (Lauckner *et al.*, 2008) so it is possible that GPR55 also regulates β -cells via G13 signalling since this G-protein has previously been identified in islets (Hammar *et al.*, 2009; Skoglund *et al.*, 1999). However, inhibition of the Rho kinase ROCK causes increased insulin release from FACS-purified rat β -cells (Hammar *et al.*, 2009; Skoglund *et al.*, 1999) and this negative role for RhoA on insulin release is

inconsistent with the observed stimulatory effects of GPR55 agonists summarised above. It is therefore possible that there are both stimulatory and inhibitory cascades activated by GPR55 agonists in islets, via separate G-proteins and further work is required to define the signalling cascades that regulate insulin exocytosis downstream of GPR55 activation.

LPI has long been known to be an insulin secretagogue (Metz, 1986), and although its effects on insulin secretion were suggested to be at least partly mediated by the mobilisation of $[Ca^{2+}]_i$ in β -cells (Metz, 1988), its precise mode of action in islets has never been established. The identification of LPI as an endogenous ligand of GPR55 (Henstridge et al., 2009a; Lauckner et al., 2008; Oka et al., 2007; Oka et al., 2009; Waldeck-Weiermair et al., 2008) points to a possibility that the insulintropic effect of LPI could be mediated through activation of this receptor. The stimulatory effects of LPI observed here in the perfusion experiments with mouse and human islets support this phospholipid being an effective insulin secretagogue, but there were some differences in the secretion profiles between species. Thus, LPI had sustained effects on basal insulin secretion from mouse islets, while it was shorter-lived in human islets, and the magnitude of the responses was greater in human islets than mouse islets. There were also strain-dependent effects such that in three separate experiments LPI failed to induce any significant effects on insulin release from C57/BL6 islets at 2mM glucose, while it consistently had effects on basal insulin release from ICR mouse islets. However, data presented in the previous chapter demonstrated that LPI was able to stimulate increases in $[Ca^{2+}]_i$ at 2mM glucose in islet cells from C57/BL6 mice. Thus, there may be differences in coupling between Ca^{2+} elevations to insulin vesicle exocytosis in ICR and C57/BL6 mice, resulting in the strain-specific secretion profiles observed here.

The capacity of LPI to stimulate insulin release from both WT and GPR55 KO mice indicate that LPI does not require interaction with GPR55 to transduce its effects on insulin exocytosis. This is consistent with the Ca^{2+} microfluorimetry data in the previous chapter showing that LPI

elevated $[Ca^{2+}]_i$ at 2mM and 20mM glucose in islet cells from both WT and GPR55 KO mice. These observations also fit with earlier reports of GPR55-independent effects of LPI in other cell types, such as acting as an intracellular agonist to stimulate voltage-gated Ca^{2+} channels (VGCCs) and Ca^{2+} -activated K^+ channels (Ben-Zeev et al., 2010; Bondarenko et al., 2010; Bondarenko et al., 2011a). If LPI also activates VGCCs in β -cells this could lead to a greater increase of $[Ca^{2+}]_i$ than that following GPR55-mediated Ca^{2+} mobilisation as a consequence of IP_3 generation, and it might help to explain why the magnitude of LPI-induced insulin secretion was greater than that of the O-1602-induced effects.

CBD is a major constituent of the cannabis plant, *Cannabis sativa*, and it is reported to be an effective GPR55 antagonist (Ryberg et al., 2007), which has been widely used to inhibit GPR55 agonist-induced biological effects (Lauckner et al., 2008; Ryberg et al., 2007; Whyte et al., 2009). However, it unexpectedly had similar stimulatory effects to O-1602 and LPI on β -cell Ca^{2+} levels and the data presented in this chapter have shown for the first time that CBD increased basal insulin secretion and potentiated glucose-induced insulin secretion from mouse and human islets. As was observed for the other agonists, species differences exist, such that CBD-induced increase in insulin release from human islets at 2mM glucose was short-lived compared to the sustained increase seen in mouse islets. Agonist effects of CBD have been observed in other studies, one of which proposed that CBD may have GPR55 agonist activity since it provided protection against acute pancreatitis in mice to a similar extent to that seen using O-1602 (Yu et al., 2013). However, perfusion studies using islets from GPR55 KO mice demonstrated that, similar to LPI, CBD does not activate GPR55 to mediate its effects in islets. These observations are in agreement with the microfluorimetry data (Section 4.3.3) showing that CBD was able to elevate $[Ca^{2+}]_i$ independently of GPR55 in islet cells, and point to agonist effects at another site(s). CBD has been implicated in the activation of a range of receptors including TRPV2 (Qin et al., 2008), 5-HT_{1A} (Campos and Guimaraes, 2008; De

Petrocellis et al., 2008; O'Sullivan et al., 2009; Resstel et al., 2009) and PPAR γ (O'Sullivan *et al.*, 2009) and CBD may mediate its stimulatory effects through these receptors.

In summary, using the sensitive perfusion technique for recording dynamic insulin secretion profiles, the results in this chapter have shown that O-1602, LPI and CBD stimulated insulin secretion from isolated mouse and human islets at sub-stimulatory (2mM) and maximal stimulatory (20mM) concentrations of glucose, although the secretion profiles varied slightly between species and mouse strain (Table 5.4.1.1). Studies using islets isolated from GPR55 KO mice demonstrated that O-1602 requires GPR55 receptor expression for its actions and LPI- and CBD-induced effects were independent of GPR55. The mechanisms through which LPI and CBD stimulate insulin secretion remain to be revealed, but they are likely to involve GPR55-independent elevations in $[Ca^{2+}]_i$.

Experimental tissues	ICR mouse islets		C57/BL6 mouse islets		Human islets	
	2mM glucose	20mM glucose	2mM glucose	20mM glucose	2mM glucose	20mM glucose
O-1602	Sustained increase	Transient increase	Sustained increase	Increase	Transient increase	Transient increase <i>N.S.</i>
LPI	Sustained increase	Increase	No effect	Increase	Increase	Increase
CBD	Sustained increase	Sustained increase	Sustained increase <i>N.S.</i>	Transient increase	Increase	Sustained increase

Table 5.4.1.1 Effects of pharmacological manipulations of GPR55 on insulin secretion.

The effects of O-1602, LPI and CBD on insulin secretion from isolated islets from ICR and C57/BL6 mice and isolated human islets has been investigated using perfusion experiments. In summary, O-1602, LPI and CBD exhibited a stimulatory effect on insulin secretion at sub-stimulatory (2mM) and maximal stimulatory (20mM) concentration of glucose from all tested experimental tissues, except that LPI failed to induce any response in islets isolated from C57/BL6 mice at 2mM glucose. *N.S.*, not significant.

In addition to stimulatory effects on second messengers such as $[Ca^{2+}]_i$ and Rho small GTPases, it has been reported that activation of GPR55 upregulated expression of the transcription factors NFAT and CREB and activated the PI3K/Akt or MAPK cell survival pathways (Andradas et al., 2011; Henstridge et al., 2009a; Henstridge et al., 2010; Kargl et al., 2013; Pineiro et al., 2011; Waldeck-Weiermair et al., 2008). GPR55 may signal through these pathways to promote islet cell survival and the next chapter will investigate the effect of O-1602, LPI or CBD on basal and cytokine-induced apoptosis in isolated mouse islets.

Chapter 6 Pharmacological manipulation of GPR55 in islets: Protection from apoptosis

**CHAPTER 6 PHARMACOLOGICAL MANIPULATION OF GPR55 IN ISLETS:
PROTECTION FROM APOPTOSIS****6.1 Introduction**

Cells undergo programmed death known as apoptosis when exposed to agents such as cytokines and reactive oxygen species (ROS). There are two main apoptotic pathways: the death receptor pathway and the mitochondrial pathway, also known as the extrinsic and the intrinsic pathway, respectively (Elmore, 2007). The perforin/granzyme pathway is suggested as an additional apoptotic pathway and some research also indicated that cells can initiate apoptosis in response to oxidative damage (Yang *et al.*, 1998; Zamzami *et al.*, 1995). The extrinsic, intrinsic and perforin/granzyme pathways signal through the same execution pathway by activating caspase-3. Activated caspase-3 triggers a chain of downstream effectors including caspase-6 and caspase-7, resulting in nuclear and protein degradations in apoptotic cells. Intracellular caspase activity is tightly regulated by a set of various pro- and anti-apoptotic molecules such as the Bcl-2 family and inhibitors of apoptosis proteins (IAPs). In addition, survival pathways such as the PI3K/Akt and MAPK pathway contribute to the regulation of apoptosis.

Increased apoptosis in β -cells has been reported in both types of diabetes and inflammatory cytokines such as interleukin-1 β (IL-1 β), tumour necrosis factor α (TNF- α) and interferon γ (IFN- γ) are key players in mediating β -cell death. Several *in vitro* studies have shown that cytokines used alone do not trigger apoptosis but their combinations induce β -cell death (Cnop *et al.*, 2005). IL-1 β , TNF- α and IFN- γ bind to their cell surface receptors on β -cells to activate downstream pro-apoptotic effectors. IL-1 β and TNF- α signal via activation of NF- κ B by phosphorylating the inhibitor of NF- κ B (I κ B) (Elmore, 2007). Activated NF- κ B translocates to the nucleus to regulate gene expression of a range of pro-apoptotic proteins. On the other hand, IFN- γ initiates apoptosis through regulation of the action of the JAK/STAT-1 pathway, which activates caspase-3 via interferon regulatory factor 1 (IRF 1) (Cnop *et al.*, 2005; Eizirik and

Darville, 2001). There are currently only a limited number of therapeutic approaches to prevent β -cell death. The GLP-1 agonist, exendin-4, was recently shown to inhibit β -cell apoptosis by activating the GLP-1 receptor (Urusova *et al.*, 2004), and it also stimulates β -cell proliferation, most likely via activation of the PI3K/Akt and MAPK pathways (Fan *et al.*, 2010; Urusova *et al.*, 2004). However, the cost, route of administration and possible side-effects of exendin-4 mean that there is considerable scope for the development of affordable and effective agents to prevent β -cell death (Singh *et al.*, 2013).

The data presented in the previous chapter have shown that GPR55 has a stimulatory effect on insulin secretion. GPR55 may also play a positive role in regulating cell survival, since studies in cancer cell lines demonstrated that its activation promotes cell proliferation via activation of survival signalling through PI3K/Akt or MAPK pathways (Andradas *et al.*, 2011; Pineiro *et al.*, 2011). However, inconsistent with those findings, another study illustrated that anandamide activates GPR55 to promote an anti-proliferative action on cholangiocarcinoma cells (Huang *et al.*, 2011). There is currently no information on the role of GPR55 in the regulation of β -cell mass, but studies in HEK 293 cells and endothelial cells have indicated that pharmacological activation of the receptor is linked to up-regulation of transcription factors (NFAT and CREB) that have been implicated in β -cell mass expansion (Henstridge *et al.*, 2009a; Henstridge *et al.*, 2010; Kargl *et al.*, 2013; Waldeck-Weiermair *et al.*, 2008). For example, NFAT has been reported to promote β -cell proliferation, while activation of CREB is essential in the GLP-1 receptor-mediated anti-apoptotic effect in β -cells (Heit *et al.*, 2006; Jhala *et al.*, 2003).

The experiments described in this chapter examined the effect of deletion of GPR55 gene expression *in vivo* on basal and cytokine-induced apoptosis in isolated mouse islets by measuring the activities of caspase-3 and -7. The effect of pharmacological manipulations of GPR55 activity on basal and cytokine-induced apoptosis of mouse islets was also investigated.

6.2 Methods

6.2.1 Measurement of caspase-3/7 activities

Groups of approximately 40-60 islets, isolated from WT and GPR55 KO mice, were incubated for 48 hours in 35mm Petri dishes containing 2ml of RPMI medium supplemented with compounds of interest under standard cell culture conditions. A cytokine cocktail (Table 2.8.1.1) was added to some groups of islets 20 hours prior to the end of the incubation period to induce apoptosis. After 48 hours incubation, 11-16 sets of 3 islets in 50 μ l medium were picked into wells of a white-walled 96-well plate. 50 μ l of Promega Caspase-Glo[®] 3/7 reagent was added to each well and the plate was mixed for 1 minute on a plate shaker. The plate was then incubated at room temperature for 1 hour before acquisition of luminescent signals using a luminometer (Section 2.8).

6.3 Results

6.3.1 Effect of GPR55 gene deletion on apoptosis in mouse islets

Basal and cytokine-induced apoptosis levels were measured in islets isolated from WT and GPR55 KO mice using the Promega Caspase-Glo[®] 3/7 Assay. It can be seen from Figure 6.3.1.1 that islets isolated from GPR55 KO mice had a significantly higher basal apoptotic level compared to islets from WT mice (percentage of increase in luminescence: 67 \pm 4%; p <0.001, n =16). 20 hours of cytokine cocktail treatment (TNF- α , IL-1 β and INF- γ) induced significant increases in caspase activities in both groups of islets (percentage of increase in luminescence: WT: 269 \pm 10%; GPR55 KO: 148 \pm 10%; p <0.001, n =16). Similar to the results obtained for basal apoptosis, islets isolated from GPR55 KO mice showed a small, but significant, increase in caspase activity in response to cytokine cocktail challenge compared to the response of islets isolated from WT mice (percentage of increase in luminescence: 16 \pm 5%; p <0.01, n =16).

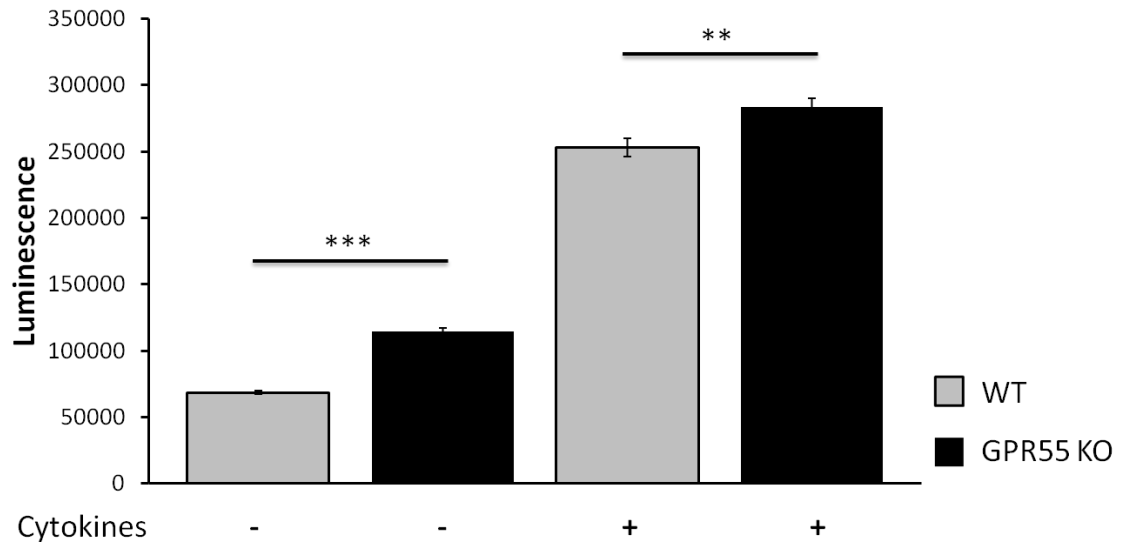


Figure 6.3.1.1 Islets isolated from GPR55 KO mice have increased apoptosis.

The presence of a cytokine cocktail (20 hours incubation) significantly increased caspase activities of islets isolated from WT mice (grey bars) and GPR55 KO mice (black bars). Islets isolated from GPR55 KO mice have higher levels of caspase activity than control islets in the absence and presence of cytokines. Data are mean \pm SEM, $n=16$ and are representative of experiments using islets isolated from 3 different groups of WT and GPR55 KO mice. ** $p<0.01$, *** $p<0.001$.

6.3.2 Effect of O-1602 and LPI on apoptosis in mouse islets

The data above demonstrating that deletion of GPR55 gene expression in mouse islets led to an increase in both basal and cytokine-induced apoptosis suggest that pharmacological activation of GPR55 would be expected to promote islet cell survival in the presence or absence of cytokines. This was demonstrated in experiments where islets from WT mice were incubated in medium supplemented with 10 μ M O-1602, 5 μ M LPI or vehicle control (0.01% DMSO) for 48 hours. As expected, O-1602 and LPI were able to significantly reduce basal apoptosis in islets (percentage reduction in luminescence signal: O-1602: 50 \pm 10%; LPI: 64 \pm 5%; $p<0.001$, $n=11$) and data shown in Figure 6.3.2.1A indicated that there was no significant difference between the effect of O-1602 and LPI ($p>0.2$). A cytokine cocktail was added to another group of islets for the last 20 hours of incubation to induce apoptosis. Consistent with the results of Figure 6.3.1.1, the cytokine cocktail induced an approximately 3-fold increase in caspase activity of the control islets (percentage increase in luminescence signal: 312 \pm 28%; $p<0.001$,

n=11, Figure 6.3.2.1B). Both O-1602 and LPI were also able to significantly ($p<0.001$) protect islets from cytokine-induced apoptosis and the protective effect of O-1602 was significantly greater than that of LPI ($p<0.001$). However, O-1602 was not able to completely protect islets from cytokine-induced apoptosis since islets pre-treated with O-1602 and exposed to the cytokine cocktail had shown a greater level of apoptosis compared to the basal apoptotic level of control islets ($p<0.001$).

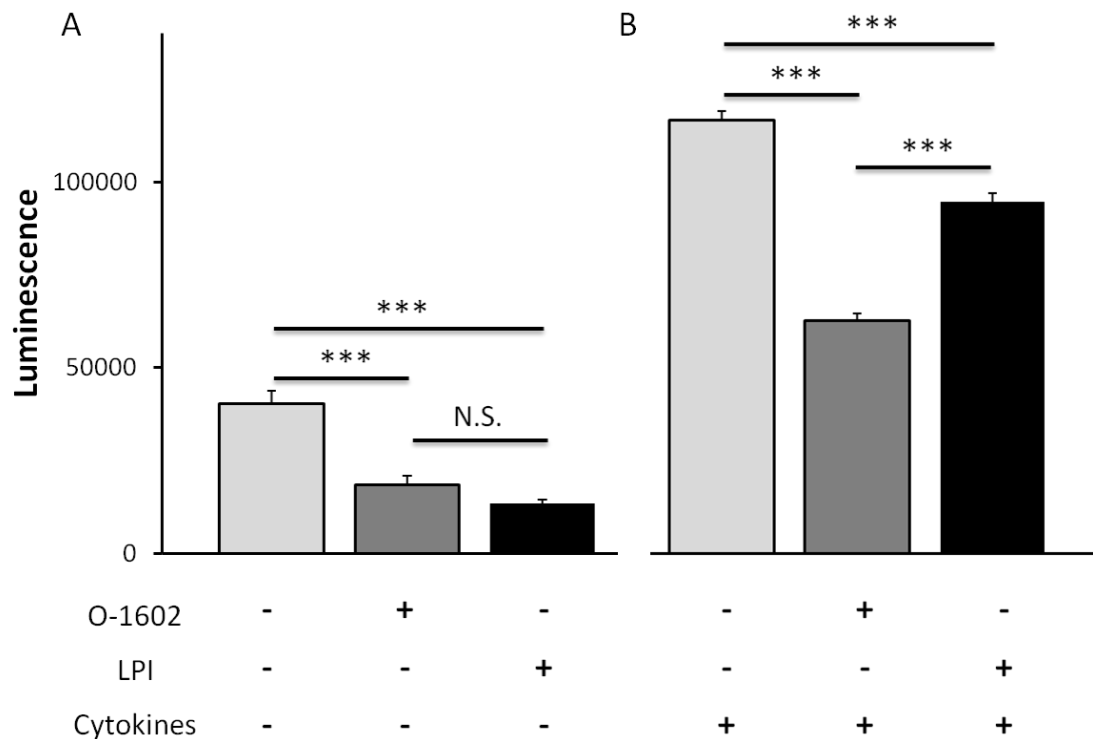


Figure 6.3.2.1 The GPR55 agonist O-1602 and putative GPR55 ligand LPI promote islet survival in the presence and absence of cytokines.

A: 48 hour treatments of islets with 10 μ M O-1602 (dark grey bar) or 5 μ M LPI (black bar) significantly reduced basal apoptosis. **B:** Both O-1602 (dark grey bar) and LPI (black bar) also protected islets from cytokine-induced apoptosis. Data are mean \pm SEM, n=11 and data are representative of experiments using islets from 5 different groups of mice. N.S., $p>0.2$, *** $p<0.001$.

6.3.3 Effect of CBD on apoptosis in mouse islets

Since pharmacological activation of GPR55 promotes islet survival (Figure 6.3.2.1) and GPR55 deletion is associated with increased islet apoptosis (Figure 6.3.1.1), it would be predicted that a GPR55 antagonist would increase apoptosis. CBD is the only commercially available GPR55 antagonist, but results presented in Chapters 4 and 5 of this thesis have indicated that it unexpectedly exerts effects in islets similar to those of O-1602 and LPI. CBD was therefore also used in islet apoptosis experiments to determine whether it had pro- or anti-apoptotic effects. It can be seen from Figure 6.3.3.1A that incubation with 1 μ M CBD for 48 hours significantly reduced basal apoptosis in mouse islets compared with islets treated with vehicle control (percentage reduction in luminescence signal: 39 \pm 6%; p <0.001, n =11). After exposure to the cytokine cocktail, islets incubated in the presence of CBD also had significantly decreased apoptosis, confirming that CBD does not demonstrate GPR55 antagonist effects in islets (Figure 6.3.3.1B). As for the experiments with O-1602 (Figure 6.3.2.1B), CBD did not completely abolish the pro-apoptotic effects of the cytokines (p <0.001 versus basal apoptosis).

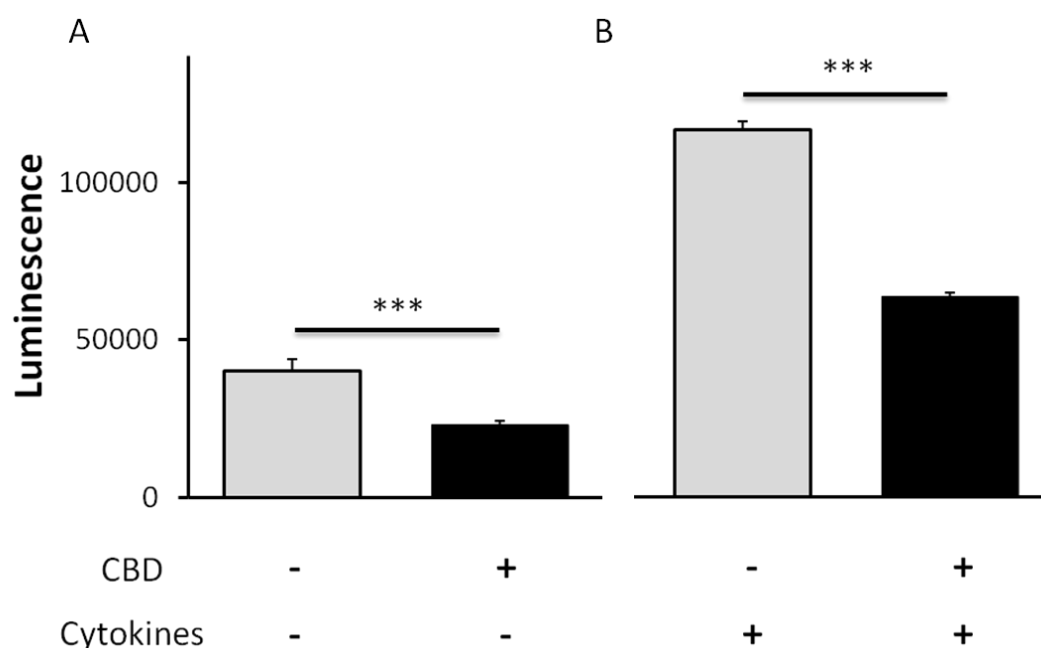


Figure 6.3.3.1 CBD promotes islet survival in the presence and absence of cytokines.

48 hours treatment of islets with $1\mu\text{M}$ CBD (black bar) significantly reduced apoptosis in the absence (A) and presence of cytokines (B). Data are mean \pm SEM, $n=11$ and are representative of experiments using islets from 4 different groups of mice. *** $p<0.001$.

6.4 Discussion

The role of GPR55 in islet survival has not been studied before, but accumulating evidence in non- β -cells has suggested that GPR55 plays a positive regulatory role in cell survival (Andradas et al., 2011; Henstridge et al., 2009a; Henstridge et al., 2010; Kargl et al., 2013; Pineiro et al., 2011; Waldeck-Weiermair et al., 2008). This chapter investigated the effects of deletion of GPR55 gene expression and pharmacological manipulations of the GPR55 receptor on basal and cytokine-induced apoptosis in isolated mouse islets by measuring capase-3 and -7 activities with a commercially available kit.

The experiments shown here have demonstrated that islets isolated from mice with global knockout of GPR55 gene expression have higher basal apoptosis in the presence or absence of

cytokines compared with islets isolated from age-matched WT mice. This pro-apoptotic effect of GPR55 is inconsistent with a study that reported an increase in osteoclast number in GPR55 KO mice (Whyte *et al.*, 2009). The explanation for this might be that GPR55 plays diverse roles in different tissues and this is supported by data from the same earlier study showing that although osteoclast number was increased following deletion of GPR55 expression, function of these osteoclasts was impaired (Whyte *et al.*, 2009). Regardless of its role in bone physiology, the observations made in this chapter suggest that GPR55 may be part of an islet self-defence mechanism against apoptosis.

Studies in GPR55-expressing HEK293 cells and endothelial cells have illustrated that pharmacological stimulation of GPR55 induced activation of the transcription factors NFAT and CREB (Henstridge *et al.*, 2009a; Henstridge *et al.*, 2010; Kargl *et al.*, 2013; Waldeck-Weiermair *et al.*, 2008). The NFAT family of proteins is regulated primarily by elevations in $[Ca^{2+}]_i$, which triggers phosphorylation of NFAT by activating calcineurin, and CREB is also a downstream target of Ca^{2+} signalling and the MAPK pathway (Hogan *et al.*, 2003; Wu *et al.*, 2001). Interestingly, data generated from cancer cell lines reported that GPR55 signalled through MAPK to promote cancer cell proliferation (Andradas *et al.*, 2011; Pineiro *et al.*, 2011). The data presented in Chapter 4 of this thesis demonstrated that O-1602 and LPI were able to increase $[Ca^{2+}]_i$ in dispersed islet cells and MIN6 cells, although O-1602, but not LPI, required the presence of the GPR55 receptor. Experiments herein have shown that the same concentrations of O-1602 and LPI as used in the calcium microfluorimetry experiments promoted islet cell survival in the absence and presence of cytokines. This suggests that O-1602 may promote islet survival by activating GPR55 to elevate $[Ca^{2+}]_i$ and downstream survival pathways as proposed in Figure 6.4.1.1. Although the effects of LPI on islet $[Ca^{2+}]_i$ were independent of GPR55, this agonist may trigger increases in $[Ca^{2+}]_i$ and inhibit islet cell apoptosis via activating cation channels or inhibiting Na^+/K^+ ATPase (Ben-Zeev *et al.*, 2010; Bondarenko *et al.*, 2010; Bondarenko *et al.*, 2011a). Inhibition of Na^+/K^+ ATPase also leads to

reduction in ROS by the mitochondria that will promote cell survival (Xie and Cai, 2003). The activity of O-1602 to protect islets against cytokine-induced apoptosis was significantly greater than the protective effect of LPI, possibly because they act on different downstream pathways.

The experiments described in this chapter have also shown that CBD protected mouse islets from basal and cytokine-induced apoptosis. Although CBD is considered to be a GPR55 antagonist, these data were as expected since CBD has been shown in this thesis to trigger elevations in $[Ca^{2+}]_i$ in islet cells, an effect that was independent of GPR55 expression (Section 4.3.2). The ability of CBD to increase $[Ca^{2+}]_i$ has also been shown in other cell types, and it may involve transient receptor potential cation channel subfamily V member 2 (TRPV2) (Mato *et al.*, 2010; Qin *et al.*, 2008). Alternatively, CBD may regulate $[Ca^{2+}]_i$ by activating peroxisome proliferator-activated receptor γ (PPAR γ) which is reported to protect islets from apoptosis by inhibiting the NF- κ B pathway (Figure 6.4.1.1) (Kim *et al.*, 2007; O'Sullivan *et al.*, 2009; Thomas *et al.*, 2007). However, studies in human breast cancer cells demonstrated that CBD has an anti-tumour effect (Ligresti *et al.*, 2006; McAllister *et al.*, 2011), suggests that it stimulates apoptosis or inhibits proliferation. There is a lack of explanation of the mode of action of CBD in these cell lines, but one study surprisingly suggested that CBD activated MAPK to reduce breast cancer cell proliferation (McAllister *et al.*, 2011).

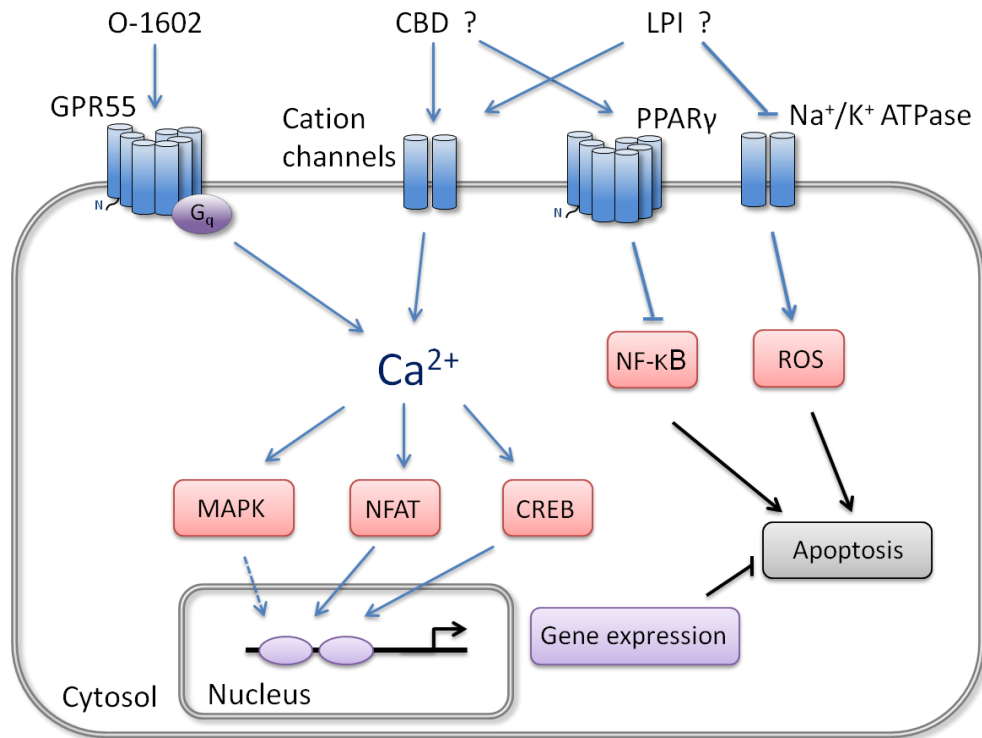


Figure 6.4.1.1 Anti-apoptotic pathways activated by O-1602, LPI and CBD.

O-1602 activates GPR55 to trigger increases in $[Ca^{2+}]_i$, which leads to activation of transcription factors NFAT and CREB and survival pathways such as the MAPK cascade, resulting in upregulation of expression of anti-apoptotic genes. It is unclear which cell surface protein(s) is activated by LPI or CBD to transduce their anti-apoptotic effects. LPI may elevate $[Ca^{2+}]_i$ via activating cation channels and it may inhibit Na⁺/K⁺ ATPase to reduce ROS-mediated cell death. CBD also regulates $[Ca^{2+}]_i$ by activating cation channels such as TRPV2. CBD may induce PPAR γ activation, which exhibits its anti-apoptotic effect by inhibiting NF- κ B.

The current results generated from global deletion of GPR55 gene expression and pharmacological manipulations of GPR55 suggest that GPR55 plays an important role to promote islet cell survival. Although the detailed signalling mechanism(s) downstream of GPR55 activation have not been established, the effect of pharmacological stimulation of GPR55 on islet cell survival is consistent with research in several other cell types. It was not possible during the course of these experiments to measure insulin secretion from islets under the same conditions that had been used for measurements of caspase-3/7 activities, but this would be of interest for future work to assess how their secretory capacity had been affected

under conditions where apoptosis had been reduced. LPI and CBD also had anti-apoptotic effects on islets, but calcium microfluorimetry experiments using islets from GPR55 KO mice (Chapter 4) have demonstrated that their actions are independent of interactions with the GPR55 receptor. Research in other cell models suggested both LPI and CBD may act on non-GPR55 cell surface proteins and further work is required to identify the correct downstream pathway(s) in islets that are responsible for the action of these two agents.

Chapter 7 General Discussion

CHAPTER 7 GENERAL DISCUSSION**7.1 Summary**

T2DM is estimated to affect more than 595 million people globally by 2035 (IDF, 2013) and there is an urgent need for new therapies to offer not only the glucose-lowering effects of existing drugs, but also the potential to delay disease progression and prevent secondary complications. Islet GPCRs are drawing strong interests for their therapeutic potentials due to the possibility of modulating GPCR activities via extracellular ligands. This has led to the launch of GLP-1R agonists for clinical treatment of T2DM since 2005 and ongoing clinical trials on other GPCR ligands, such as free fatty acid receptor 1 agonist, TAK-875 (Araki et al., 2012).

The search for novel T2DM therapeutic targets continues and, as summarised in Section 1.3, recent studies in islet biology have identified the roles of two GPCRs, CB1 and CB2, in regulating islet hormone secretion. However, there is currently a lack of consensus in terms of the expression and function of CB1 and CB2 in islets (Di Marzo, 2008; Li et al., 2010), possibly due to different experimental settings (Anderson et al., 2013) or off-target effects of some cannabinoid ligands (Curran et al., 2005; Derocq et al., 1998; Kaplan et al., 2005; Nieri et al., 2003). Reports published since 2007 have suggested that GPR55 may be a third cannabinoid receptor as it can be activated by a range of endogenous and pharmacological cannabinoids (Ross, 2009; Sharir and Abood, 2010). It has been shown that activation of GPR55 resulted in elevations in $[Ca^{2+}]_i$ in GPR55-HEK293 cells, cancer cell lines, primary endothelial cells, presynaptic cells and cardiomyocytes (Henstridge et al., 2010; Kargl et al., 2013; Oka et al., 2007; Pineiro et al., 2011; Sylantsev et al., 2013; Waldeck-Weiermair et al., 2008; Yu et al., 2013), suggesting the possibility that it may also be coupled to Ca^{2+} mobilisation and insulin secretion in β -cells. In addition, GPR55 has been shown to activate transcription factors such as CREB and NFAT (Henstridge et al., 2010; Henstridge et al., 2009b; Oka et al., 2010) and to promote proliferation in cancer cells (Andradas et al., 2011;

Perez-Gomez et al., 2013; Pineiro et al., 2011). This implies that activation of GPR55 could help to maintain or increase β -cell mass, if similar pathways exist in the endocrine pancreas. In order to investigate whether GPR55 is a potential therapeutic target for providing glycaemic control and β -cell protection for T2DM patients, the experiments described in this thesis examined the expression and functional roles of GPR55 in islets of Langerhans.

As described in Chapter 3, expression of GPR55 mRNA and protein was initially examined and data from these experiments indicated that GPR55 was expressed in MIN6 β -cells, and also in mouse and human islets. Experiments presented in Chapter 4 were then performed to investigate whether GPR55 is coupled to second messenger generation in β -cells. The single-cell calcium microfluorimetry experiments revealed that pharmacological activation of GPR55 led to elevations in $[Ca^{2+}]_i$ in MIN6 cells, as well as in dispersed mouse and human islet cells. On the other hand, GPR55 was shown not to be coupled to RhoA, a member of Rho GTPase family, in MIN6 cells. Although we cannot rule out that GPR55 may be linked to other Rho GTPases, evidence presented here strongly suggests that GPR55 is coupled to the Gq subunit to stimulate Ca^{2+} mobilisation in β -cells. This led us to test whether activation of GPR55 is associated with increased insulin secretion from β -cells. Dynamic insulin secretion from mouse and human islets was thus measured in the perfusion experiments described in Chapter 5, and the data obtained demonstrated that activation of GPR55 stimulated basal insulin secretion and potentiated glucose-induced insulin secretion from both mouse and human islets. The stimulatory effects of GPR55 agonist were lost in islets isolated from GPR55 KO mice. Taken together, the data presented in Chapters 4 and 5 suggest that GPR55 plays an important role in regulating insulin secretion from β -cells. In Chapter 6, the capacity of GPR55 to maintain functional islet mass was examined. It was found that GPR55 deletion resulted in higher caspase activity in mouse islets and activation of GPR55 protected them from undergoing apoptosis, consistent with GPR55 also playing a role in regulating β -cell mass.

The data generated during this thesis are consistent with those reported in two recent publications investigating the role of GPR55 in rodent islets (McKillop et al., 2013; Romero-Zerbo et al., 2011). Data from the perfusion experiments described here provide robust measurements of dynamic, minute to minute changes in insulin release in response to GPR55 agonists, which further extend our understanding of their effects on insulin secretion obtained from the static incubation experiments performed by others (McKillop et al., 2013; Romero-Zerbo et al., 2011). Immunofluorescence staining data presented in those studies suggested that GPR55 was only expressed by insulin-expressing β -cells, consistent with a functional role of GPR55 in regulating β -cell functions. It was also shown that activation of GPR55 by O-1602 and other GPR55 agonists stimulated increases in $[Ca^{2+}]_i$ and insulin secretion from β -cell lines and rodent islets. The *in vivo* studies reported in those papers also demonstrated that O-1602 administration increased plasma insulin levels and improved glucose handling in rodents (McKillop et al., 2013; Romero-Zerbo et al., 2011). Thus, all studies to date, including the research described in this thesis, indicate that GPR55 is expressed by islet β -cells and it is coupled to Ca^{2+} mobilisation and insulin secretion.

The signalling pathways downstream of GPR55 in β -cells have not been defined, although experiments in other cell types have shown that GPR55 is coupled to either Gq or G12/13 subunits (Henstridge et al., 2009b; Lauckner et al., 2008; Ryberg et al., 2007). Data presented in this thesis and in other studies indicate that GPR55 activation leads to Ca^{2+} mobilisation in β -cells, implying that GPR55 is coupled Gq. G12/13 is coupled to RhoA and its effector protein ROCK (Obara et al., 2011; Ryberg et al., 2007; Whyte et al., 2009), and both RhoA and ROCK have been shown to play a negative role in insulin secretion (Hammar et al., 2009). The RhoA assay described in Chapter 4 demonstrated that although glucose stimulated RhoA activation after 10 minutes of incubation, activation of GPR55 with O-1602 was without effect, suggesting that the receptor is not linked to the RhoA-ROCK cascade in islets. However, it would be useful in future experiments to determine whether RhoA is activated by O-1602 in β -

cells over an extended time-course by performing the activation assay over 1-30 minutes. Only RhoA activation was measured in these experiments and the relationship between GPR55 and other Rho small GTPases was not tested, so the possibility that GPR55 is coupled to G12/13 in β -cells cannot be ruled out. In addition, activation of GPR55 is also reported to induce cAMP generation in β -cells (McKillop et al., 2013). Since there is no evidence that GPR55 is coupled to Gs, it is possible that GPR55 can regulate cAMP through modulating Ca^{2+} -sensitive AC isoforms, secondary to elevations in $[\text{Ca}^{2+}]_i$.

Experiments described here investigated, for the first time, the expression and function of GPR55 in human islets and obtained findings consistent with observations from rodent studies. Thus, data from Chapter 3 demonstrated that GPR55 is expressed by human islets and experiments in Chapters 4 and 5 have shown that activation of GPR55 stimulated elevations in $[\text{Ca}^{2+}]_i$ in dispersed human islet cells and insulin secretion from human islets at both sub-stimulatory and stimulatory concentrations of glucose. It is worth noting that insulin secretion profiles recorded from human islets following GPR55 ligand administration were different from those obtained using mouse islets, perhaps highlighting species variability in islet biology (Cabrera et al., 2006).

O-1602 is reported to be a potent and selective GPR55 agonist that lacks significant binding affinity for either CB1 or CB2 (Johns et al., 2007; Ryberg et al., 2007; Waldeck-Weiermair et al., 2008). By using islets isolated from WT and GPR55 KO mice, experiments in this thesis confirmed that the effects of O-1602 in islets are dependent on GPR55 expression and that O-1602 is a selective agonist for stimulating GPR55 activity in islets, as had been reported earlier in static incubation studies. (Romero-Zerbo et al., 2011). The bioactive lipid, LPI has also been suggested by some studies to be a specific endogenous ligand for the receptor in various cell lines (Henstridge et al., 2009b; Lauckner et al., 2008; Oka et al., 2007; Oka et al., 2009; Waldeck-Weiermair et al., 2008) and in primary cells (Kargl et al., 2013; Pineiro et al., 2011;

Sylantiev et al., 2013). However, the stimulatory effects of LPI on Ca^{2+} mobilisation and insulin secretion were observed in islets from both WT and GPR55 KO mice, as shown in Chapter 4 and 5, respectively. This suggests that LPI does not depend on GPR55 to mediate its physiological effects in islets and it may activate Ca^{2+} and/or K^{+} channels instead (Bondarenko et al., 2010; Bondarenko et al., 2011a, b). Furthermore, the phytocannabinoid CBD is generally considered to be a GPR55 antagonist (Lauckner et al., 2008; Ryberg et al., 2007; Whyte et al., 2009) and it has been reported that CBD was able to attenuate GPR55 ligand-induced insulin secretion from BRIN-BD11 cells and mouse islets (McKillop et al., 2013). In contrast to these findings, data presented in Chapters 4 and 5 demonstrated that CBD exerted similar stimulatory effects to O-1602 in islets, in both the presence and absence of GPR55 gene expression. The exact mechanism of action of CBD in islets remains unknown, but studies in other cell types have suggested that it may activate TRPV2 cation channels (Qin et al., 2008) or PPAR γ (Shimomura et al., 2004). There are studies currently developing novel, selective GPR55 agonists and antagonists and promising candidates have recently been identified using β -arrestin recruitment assays for high-throughput screening of compound libraries (Kotsikorou et al., 2011; Kotsikorou et al., 2013) or of coumarin derivatives (Rempel et al., 2013). These competitive GPR55 ligands, which have negligible pharmacological effects on CB1 and CB2 receptors, have much potential for investigating whether GPR55 may be a suitable drug target, but their effects on biological functions are largely untested. However, promising data have been obtained with a novel antagonist, CID16020046, which effectively reduced GPR55 agonist-mediated physiological responses in primary human platelets and endothelial cells (Kargl et al., 2013). These agents are likely to be useful tools for future studies in the role of GPR55 in islet function (see Section 7.2).

The experiments described in Chapter 6 were the first measurements of the effects of GPR55 on islet cell apoptosis. A luminescence caspase assay optimised by our group for measuring islet cell apoptosis was employed to assess the caspase-3 and -7 activities of islets in the absence

and presence of a cytokine cocktail, which was used to induce apoptosis in islets. Data presented in Chapter 6 have shown that islets from GPR55 KO mice had higher basal and cytokine-induced caspase activities compared to those from WT mice. In addition, 48 hours exposure to O-1602 protected mouse islets from undergoing apoptosis. These observations are consistent with an anti-apoptotic signalling cascade downstream of GPR55 in islet cells and with publications indicating GPR55 is coupled to the MAPK cell survival pathway and the transcription factors NFAT and CREB (Henstridge et al., 2010; Henstridge et al., 2009b; Kargl et al., 2013; Oka et al., 2010; Waldeck-Weiermair et al., 2008). As described in Chapter 1, MAPK, NFAT and CREB play important roles in the regulation of β -cell survival. GPR55 may therefore play a role in promoting β -cell survival, which is an area where further research is required (see Section 7.2). In addition, both LPI and CBD were also able to protect islets from undergoing apoptosis, but time constraints meant that it was not possible to determine whether these effects were via GPR55 activation or occurred independently of GPR55. Further studies could measure insulin secretion from these islets after treatment with cytokines in the absence and presence of GPR55 agonists to determine whether their insulin secretory capacities are improved after GPR55 activation. Further work aimed at defining their mechanisms of action opens up the possibility of identifying novel therapeutic targets to maintain functional β -cell mass in T2DM patients.

Thus, the main observations of this thesis are illustrated schematically in Figure 7.1.1 and can be summarised as follows: the novel cannabinoid receptor GPR55 is expressed by MIN6 β -cells and by mouse and human islets at both mRNA and protein levels. GPR55 is coupled to elevations in β -cell $[Ca^{2+}]_i$, but not to RhoA activation. In addition, activation of GPR55 leads to enhanced insulin secretion and promotes β -cell survival in islets. Unlike CB1 and CB2, all studies to date indicate that activation of GPR55 stimulates insulin secretion, and the observations here, of reduced apoptosis following GPR55 activation, suggest that GPR55 may be a target for the development of novel treatments for improving glycaemic control in

individuals with T2DM. However, since activation of GPR55 stimulates insulin secretion at both basal and stimulatory glucose concentrations exposure to GPR55 agonists *in vivo* may have the side effect of hypoglycaemia, as may occur with sulphonylurea therapy. On the other hand, GPR55 may also be a target to maintain and/or increase β -cell mass to delay the progression of T1DM and T2DM, but a potential side effect of this is increased cancer risk in GPR55-expressing tissues, such as bone, GI tract, adrenal and spleen. Further studies in tissues in addition to islets will further help us design and develop safe and effective therapeutic approaches targeting GPR55 for treating diabetes.

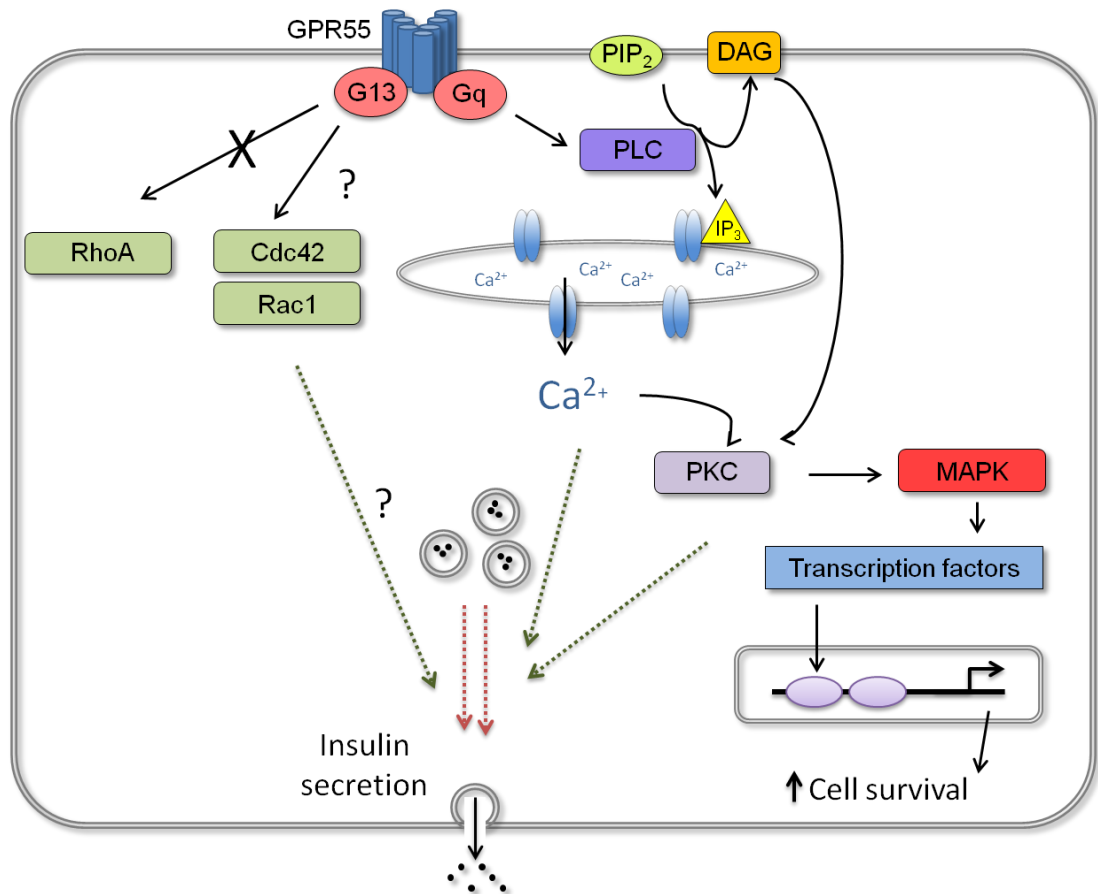


Figure 7.1.1 Schematic summary of the signalling pathways downstream of GPR55 in the regulation of β -cell function.

Activation of β -cell GPR55 causes elevations in $[\text{Ca}^{2+}]_i$, possibly via PLC-induced Ca^{2+} release from the ER. PKC is also activated following PLC-induced DAG generation and together with increases in $[\text{Ca}^{2+}]_i$ activated PKC stimulates insulin secretion. In addition, PKC can also modulate β -cell mass by regulating MAPK and transcription factors (such as CREB and NFAT). Furthermore, GPR55 is not coupled to RhoA in islets and it is currently unknown if it is coupled to G12/13 and other Rho small GTPases to regulate β -cell function.

7.2 Further studies

Experiments described in this thesis provide a firm foundation on the role of GPR55 in the regulation of insulin secretion and cell survival in islets of Langerhans. However, due to time constraints it was not possible to fully define the role of this receptor in islets. Future work should first aim to improve the robustness of the current findings from this thesis. This will include performing further perfusion experiments to investigate how islets isolated from WT and GPR55 KO mice respond to 20mM glucose over time-courses longer than 30 minutes, to help us confirm that the potentiation of glucose-induced insulin secretion observed in Section 5.3 is due to the action of the test compounds. In addition, understanding of how O-1602 regulates the activity of RhoA at other time points and the activities of other Rho small GTPases (e.g. Cdc42 and Rac1) will clarify if GPR55 is coupled to G12/13 in β -cells. Furthermore, further work is required to investigate whether the anti-apoptotic effects of O-1602, LPI and CBD are abolished in islets from GPR55 KO mice. It will also be interesting to measure insulin secretion from islets under the conditions tested in the caspase-3/7 apoptosis assays to determine if insulin secretory responses are improved under conditions where apoptosis is decreased. In order to gain a full picture of the role of GPR55 in islet function experiments should also be performed to investigate the effects of GPR55 agonists and antagonists on glucagon and somatostatin secretion.

Studies so far have mainly focused on understanding the roles of GPR55 in rodent islets, but it will be more clinically relevant to translate these findings, where possible, to human islets. Therefore, further studies should be performed using human islets to address which endocrine cells express GPR55, to examine whether O-1602 protects human islets from undergoing apoptosis and to investigate the effects of selective GPR55 agonists and antagonists on glucagon and somatostatin secretion from human islets.

Further studies should also address the relationship between GPR55 and CB1 and CB2 receptors in islets to gain understanding of the possible crosstalk between GPR55 and the ECS. One study in endothelial cells has demonstrated that the CB1 and GPR55 signalling cascades exhibit negative feedback on the other, a process that depends on the status of integrin configuration (Waldeck-Weiermair et al., 2008). Another recent publication has indicated that GPR55 and CB1 receptors alter each other's signalling properties at the levels of MAPKs and downstream transcription factor activation (Kargl et al., 2013). GPR55 was also shown to regulate CB2-mediated responses in human neutrophils through Rac1 and Cdc42 (Balenga et al., 2011). Potential cross-regulation between the three receptor systems in islets would have to be taken into consideration when exploring the therapeutic potential of targeting GPR55.

Research into the roles of GPR55 in glucose homeostasis can further benefit from *in vivo* studies. The most logical experiments are studying the effects of administration of GPR55 agonists and antagonists on plasma insulin, glucagon and glucose levels in WT and GPR55 KO mice. Further studies may also examine the effects of GPR55 receptor stimulation and antagonism on β -cell proliferation and apoptosis in WT and GPR55 KO mice *in vivo*. These studies will help us to determine the therapeutic potential of targeting GPR55 for regulating glucose homeostasis and maintaining β -cell mass.

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